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FEASIBILITY OF COMMERCIAL SPACE MANUFACTURING

Production of Pharmaceuticals

FINAL REPORT

Volume III
Product Data

MCDONNELL DOUGLAS ASTRONAUTICS COMPANY-ST. LOUIS DIVISION

MCDONNELL DOUGLAS CORPORATION

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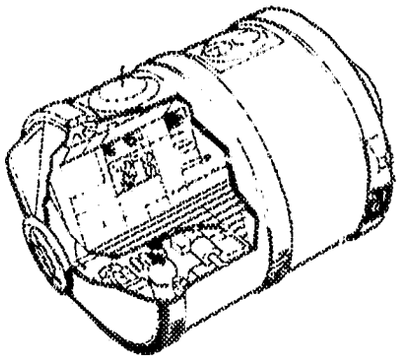
9 November 1978



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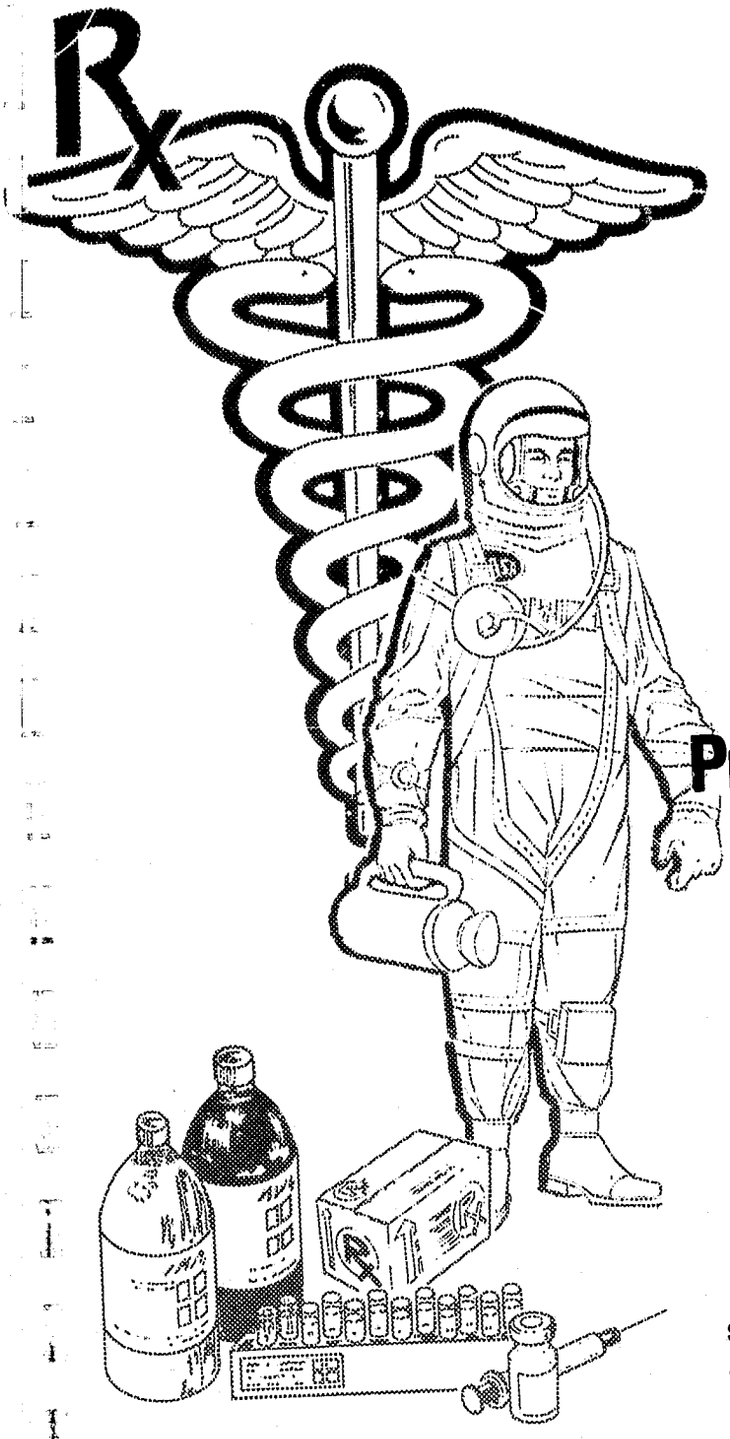

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Production of Pharmaceuticals

FINAL REPORT

Volume III
Product Data

SUBMITTED TO NATIONAL AERONAUTICS AND SPACE ADMINISTRATION MARSHALL SPACE FLIGHT CENTER HUNTSVILLE, ALABAMA UNDER CONTRACT NO: NAS 8-31353

MCDONNELL DOUGLAS ASTRONAUTICS COMPANY-ST. LOUIS DIVISION

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MCDONNELL DOUGLAS CORPORATION

PREFACE

This report describes the study results achieved over two periods of related activity, June-December 1977 and March-October 1978. It is organized in three volumes to meet the needs of different audiences. The first, an executive summary, serves as an overview aimed at those responsible for committing public and private resources to new ventures. The second recounts the activities of the study and presents fundamental lessons learned. This volume is intended to serve two groups: those in the aerospace industry who may wish to have a model for their efforts to attract participation in space processing by other industries; and those in nonaerospace industries who want to learn more about the possibilities of space processing. The third volume contains the detailed product data collected and reviewed to support the activities in Volume II.

The report has been organized to take the reader through the chronology of the study process. Although many of these steps were accomplished simultaneously, we have -- for simplicity and clarity -- organized them into discrete segments, moving first through the plan established to target and contact pharmaceutical companies, then the laboratory work needed to support the expanding company-to-company cooperation and technology interchange, through the literature search and analysis of potential products and finally through the production engineering analysis. The report then summarizes the study by identifying the lessons learned during the course of its execution. Before the enormous potential benefits of space processing can reach the public -- the basic goal of NASA's Materials Processing in Space program -- industry must be willing to participate in the development of processes. Such investment, however, will not follow until industry itself is made aware of the promise of space processing and is supplied with hard data supporting such promises. It is to this purpose that we have directed our efforts.

This report is submitted under NASA Contract Number NAS8-31353. The work was performed by McDonnell Douglas Astronautics Company - St. Louis Division under the direction of William R. Marx and Dr. Ronald A. Weiss, Study Managers during the first and second periods of the study respectively. This contract was administered by the NASA Marshall Space Flight Center, Huntsville, Alabama.

This report was prepared under the direction of Dr. Ronald A. Weiss with the assistance of the principal contributors listed below:

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FOREWORD

As the principal result of this study activity, the McDonnell Douglas Astronautics Company - St. Louis Division, elicited and fostered the participation of several pharmaceutical firms, to varying degrees, in exploring the potential benefits which may accrue from processing pharmaceuticals in space.

One of the conditions for their participation, however, was that the companies not be linked with any potential product or process because of the highly competitive nature of the industry. With NASA concurrence, therefore, and participating company approval, we have deleted the names of any company associated with this study in order to be able to emphasize the important product data and technology interchange achieved with them.

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NAME (GENERIC): Alpha-1-Antitrypsin
Alpha-Trypsin Inhibitor
Alpha-Protease Inhibitor

(PROPRIETARY): None

DISEASE TREATED: Emphysema

PATIENTS: Alpha-1-antitrypsin probably functions as a major control protein against the tissue damaging effects of both endogenous and exogenous enzymes. It is the major inhibitor of two enzymes from polymorphonuclear granulocytes which have been found to have the ability to destroy human pulmonary tissues (35, 36) resulting in emphysema. Abnormally low levels of plasma alpha-antitrypsin are found in some families with multiple generation histories of emphysema and/or infantile cirrhosis (37, 38). Some investigators feel that replacement therapy might prove of value in patients having a genetic antitrypsin deficiency.

Usually emphysema or chronic obstructive pulmonary disease begins near the end of the third decade in life (37) subtly starting with an increase in respiratory effort. The disease progresses rapidly, showing diffuse destruction of elastic tissue, rupture and recoil of the alveolar walls. Recurrent infections with bronchitis and severe bronchiectasis may become superimposed upon the diffuse underlying pathological process. The progression of emphysema can be clearly hastened by smoking or by environmental insults to the pulmonary system.

A few children with the disease have been reported (4) but it is believed the disease was triggered by asthma, cystic fibrosis or an infectious disease within the pulmonary system.

The National Disease and Therapeutic Index indicated that 800,000 people in the United States were diagnosed as having emphysema or chronic bronchitis. Because of the wide disparity in the literature with regard to patients afflicted with both alpha-antitrypsin deficiency and emphysema or chronic obstructive pulmonary diseases, we have conservatively estimated that approximately 100,000 people of this group have a severe alpha-antitrypsin deficiency, usually less than 20% of normal circulating blood levels. Some surveys estimate at least three percent of

all persons over forty have early signs of obstructive lung disease while other surveys estimate that twenty-five percent of the population over forty may have some degree of impaired function. Davis (5) reports there were 26,000 deaths in the United States from emphysema in 1974 and the rate appears to be doubling every five years.

Alpha-antitrypsin may also hold some therapeutic benefit to persons suffering from osteogenesis imperfecta, Marfan's syndrome and cutis laxa. Each of these diseases has an underlying pathology related to destruction or disruption of elastic fibers attacked by trypsin. The number of patients in each category is very low, probably less than several thousand.

SEVERITY ESTIMATE: Those people who are afflicted with emphysema can have symptoms that range from chronic mild physiological disfunction to chronic debilitating conditions where the patient is no longer capable of employment or performing the simplest physical tasks. Between these two extremes, there are episodes of acute debilitation.

TREATMENT REGIMEN: 50 mg/Kg body weight for initial treatment followed by half that dose at four to six day intervals. Values are derived from work of Makino and Reed (6).

TREATMENT METHOD: Makino and Reed have reported that the normal serum levels for 1063 men between 15 and 40 years of age is 310 mg/100 ml of plasma with a range of 233-422 mg/100 ml (6). The mode for 1390 women of the same age is 336 mg/100 ml. Patients with hereditary alpha-antitrypsin deficiency are not completely devoid of alpha-antitrypsin but usually have about 5-20 percent of normal levels. These investigators have suggested that partial replacement of this protein to levels about half normal might be useful for these patients.

Because it is a protein, alpha-antitrypsin must be injected directly into the blood stream. The half life of this protein, in the circulation of patients completely devoid of any alpha-antitrypsin, is 5-6 days (6). Kueppers and Fallat (39) using radio labelled alpha-antitrypsin have shown that the half life of this protein is the same for controls as well as six emphysematous patients, usually being about four days.

With such a relatively long half-life, Makino and Reed have suggested that after one-half the normal concentration of alpha-antitrypsin is established in the patients circulation, it can be maintained at that level by periodic (every 4-6 days) intravenous injections of purified alpha-antitrypsin at half the initial priming dose. This is essentially the same procedure used to treat hemophilic patients with antihemophilic factor VIII.

CURRENT GROUND STATUS: No specific treatment now exists for a severe alpha-antitrypsin deficiency. It was first observed in 1963 during routine plasma electrophoresis of patients by Laurell and Eriksson (20). It very rapidly became obvious that this deficiency was family related and highly correlated with chronic lung disease (37). Fagerhol and Laurell demonstrated that this protein deficiency was inherited through a series of co-dominant allelic genes (19) which seem to control both the electrophoretic mobility and serum concentration of alpha-antitrypsin. This protease inhibitor (Pi) system is known to have at least 24 alleles (40) which can be identified by various electrophoretic methods. These alleles have been named alphabetically according to their mobility with the M variant being the most common allele, 0.87-0.99 gene frequency in various populations, and producing "normal" amounts of alpha-antitrypsin. The Z variant is the slowest moving alpha-antitrypsin with a gene frequency of 0.1-2.2. People with this variant usually present only 10% of the "normal" amount of alpha-antitrypsin on blood analysis. The F variant, with a gene frequency of 0.003-0.09, is the fastest moving alpha-antitrypsin and is usually found in concentrations equal to the M variant. All other variants show alpha-antitrypsin concentrations and mobilities intermediate between the M and Z variant (4). Those having the S or Z variant stand to benefit the most from A-AT replacement therapy.

By a recent (41) international agreement the phenotype of the homozygous individuals will be represented as Pi M, Pi Z, Pi S, etc. unless a genetic study has confirmed the presence of two similar alleles. In that circumstance the two alleles will be identified as, for example, Pi MZ.

Early investigation of the use of alpha-antitrypsin as a therapeutic agent was accomplished by the transfusion of whole human plasma containing many more proteins than the alpha-antitrypsin. As much as ten units of plasma were required

for the initial dose to bring the patient's alpha-antitrypsin levels to half normal values. Because of the tremendous quantities of fluid and foreign protein infused on a routine basis, pathological problems rapidly developed which negated the therapeutic effect for emphysema.

The fluid quantities infused could be drastically reduced if the alpha-antitrypsin could be isolated and highly purified to remove most of the contaminating proteins. The present isolation and purification procedures are very time consuming (usually about ten days to a final product) and result in very low yield for the quantities required. These considerations plus the high cost for the raw plasma and processing chemicals have made therapeutic alpha-antitrypsin commercially unfeasible at this time.

Two other approaches to accomplishing the same therapeutic objective are currently being explored. Since alpha-antitrypsin is produced in the liver cells, but not released in patients with emphysema and cirrhosis, induction of its release may lead to higher circulating levels. Although a number of compounds have been tried to stimulate release, to date none has worked. The second approach is to use synthetic inhibitors of granulocyte elastase and trypsin. Tests are being conducted in vitro but these chemical compounds appear to be toxic to cells.

An important consideration in the use of pharmacologicals, even of natural origin, in the prophylaxis of emphysema is the fact that a few Z variant individuals in families with a history of emphysema never develop clinically significant pulmonary symptoms although pulmonary function tests may demonstrate a subclinical abnormality. Any long term or potentially harmful treatment would require careful consideration before being implemented under these circumstances (4).

b) STARTING MATERIAL, PRODUCTION AND FINISHED PRODUCT: At the current time all alpha-antitrypsin used in research is obtained from human plasma. There are two major methods for the isolation and purification of this product (8, 21) but each depend on the use of both the "salting out" phenomenon and affinity chromatography to remove impurities. The main impurity in plasma is albumin which has a very similar solubility to alpha-antitrypsin. Albumin is usually removed by passing

the plasma through an affinity chromatography column consisting of either Sepharose coupled to antibodies against albumin or Sepharose 4B coupled with blue dextran (Cibacron).

Moser and co-workers (21) used the following procedure to purify alpha-antitrypsin from 125 ml of human plasma treated with acid-citrate-dextrose to prevent clotting. All work had to be done at 4°C.

1. Add equal volume of saturated ammonium sulfate solution to starting plasma and allow to stand overnight.
2. Centrifuge mixture for 15 minutes at 10,000g to remove salt.
3. Decant supernatant.
4. To every 100 ml of supernatant collected add 17.2 gms of solid ammonium sulfate (ultrapure) while stirring for one hour.
5. Centrifuge for 15 minutes at 10,000g and discard the supernatant.
6. Dissolve precipitate in minimal volume of 0.05M TRIS buffer (pH = 8.6) with 0.05 NaCl.
7. Dialyse mixture against one liter of buffer for 12 hours.
8. Repeat step 7 twice with a change in external buffer each time.
9. Apply the remaining material in the dialysis bag to a QAE-Sephadex column.
   NOTE: This 2.6 x 40 cm column was previously equilibrated with the 0.05 M tris buffer.
10. Elute the alpha-antitrypsin with a linear gradient of the same buffer.
    NOTE: This gradient is produced by flowing 400 ml of the 0.05M tris buffer into a constant volume chamber containing an equal volume of a solution composed of 0.05M tris buffer and 0.35 M NaCl.
11. Collect the eluted fluid in 5 ml fractions at the rate of 0.5 ml per minute.
12. All fractions having a trypsin inhibitory capacity greater than 0.1 mg/ml are pooled and concentrated by adding 5.16 gms solid ammonium sulfate/10 ml and centrifuged at 10,000g for 15 minutes.
13. The resultant precipitate containing alpha-antitrypsin and salt is again dissolved in a minimal volume of tris buffer (pH = 8.0) containing 0.1M NaCl.
14. Dialyse the mixture of step 13 against 750 ml of the buffer for 12 hours.

15. Repeat step 14 a second time with a change in external buffer.
16. The dialysate is passed through a 0.45 μ Millex filter.
17. The collected dialysate is then applied to a Concanavalin-A Sepharose 4B column.

    NOTE: This 1.6 x 40 cm column was previously equilibrated with a solution consisting of 0.05M tris buffer (pH = 8.0), 0.1M NaCl, 0.001M $MgCl_2$ and $CaCl_2$.
18. Wash the column with two column volumes of the equilibration buffer to remove the unbound protein.
19. Elute the bound alpha-antitrypsin using the equilibration buffer supplemented with 0.2M alpha methyl glucoside flowing at 0.6 ml/minute.
20. Collect the eluate in 8 ml fractions.
21. Fractions indicating an alpha-antitrypsin inhibitory capacity of greater than 0.4 mg/ml or 0.2 mg/ml for the Z variant are pooled and again concentrated by repeating step 12.
22. The centrifuged precipitate is resuspended in a minimal volume of 0.05M tris (pH = 7.4) with 0.1M NaCl.
23. Dialyse the mixture from step 22 against 500 ml of buffer for 12 hours.
24. Repeat step 23 with fresh buffer external to the dialyzing membrane.
25. Pass the sample through a 0.45 micron Millex filter.
26. Store the filtrate at -20°C.

The above procedure takes approximately 10 days to perform. It provides about 90% purity and the alpha-antitrypsin is both biologically and phenotypically intact as determined by the Oucterlony method, immunoelectrophoresis and polyacrylamide gel electrophoresis. The solution is considered sterile because of the filtration steps involved. Miller and co-workers (1) found the need to add 0.001M beta mercaptoethanol in all the buffer as a biocide to prevent loss of biological activity.

The other major purification method used by Jeppsson and Laurell (8) is essentially the same up to step nine of the above method. At that point the dialyzed mixture is applied to a Sepharose 4B column conjugated with rabbit IgG anti-alpha-antitrypsin. Elution is accomplished with 3M sodium thiocyanate. This second method gives a reportedly pure material but the immunoreactivity to its corresponding antibodies is diminished.

c) YIELD: The yield of α-1 antitrypsin will vary depending on the laboratory techniques currently employed. In general, it does not exceed 40% of the original starting value (1, 2, 3). Most of the loss can be attributed to the protein being permanently bound to an affinity chromatography column or being trapped in the first ammonium sulfate precipitation step. The usual laboratory yield, however, is about 22% of that found in crude plasma.

d) PURITY: Commercially available α-1 antitrypsin for research or diagnostic purposes is derived from pooled human plasma. It usually contains about 20% antitrypsin activity with albumin, α_1 globulins, α_2 macroglobulins, chymostrypsin inhibitor and β mercaptoethanol as impurities depending on the procedure used for preparation of the material. There are 24 known genetically related modifications of this protein. This results in an electrophoretic microheterogenicity of the final product when serum is collected from many individuals and used as the pooled starting material. The electrophoretic mobilities of these modified proteins is sufficient to provide separation into its allele forms (4). Alpha-1-antitrypsin can be obtained in greater than 95% purity (5) when collected from a single individual using current laboratory procedure.

e) COST/DOSE (to patient) AND AVAILABILITY: Partially purified alpha-antitrypsin is available for laboratory reserach and diagnostic purposes at $0.17 to $0.20/mg. There are two American and one German companies that produce alpha-antitrypsin for these purposes. They are Sigma Chemical Company, St. Louis, Missouri; Worthington Biochemical Corporation, Freehold, New Jersey; and Behringwerke, Darmstedt, Germany. Human material as an alpha-antitrypsin standard can also be obtained from Behring Diagnostics, Woodbury, New York. This protein is not available as a purified, or partially purified, clinical product from a pharmaceutical company or government research facility. In some cases human plasma, known to contain alpha-antitrypsin, has been used as the source of this product for investigations involving human patients with antitrypsin deficiencies.

f) STORAGE: Isolated, lyophilized alpha-antitrypsin is stable, when stored at -20°C, for at least four months and can be maintained indefinitely in a saturated ammonium sulfate solution at 4°C provided mercaptoethanol is present (7). Worthington Biochemical Corporation advertizes that their human alpha-antitrypsin in a salt free, lyophilized form is stable for 12-18 months when stored at 5°C.

TECHNICAL DATA

a) MOLECULAR WEIGHT: Twenty-four alleles of this protein have been determined using electrophoresis (9). Most investigators agree that the average molecular weight of this protein is about 50,000 but values in the literature range from 42,000 (7) to 55,000 (8). Since molecular weight determinations derived empirically by SDS gel electrophoresis are not reliable for glycoproteins (10), ultracentrifugation values based on sedimentation velocity have been determined by Roll and his associates (11). The M variant has a molecular weight of 47,300 while both the S and Z variant have a molecular weight of 47,500. Miller and his group (1) agreed on the weight of the Z variant but reported 46,700 as the weight of the M variant. Since the actual chemical sequencing of this molecule has not been accomplished these weight values must be considered approximate.

b) MOLECULAR STRUCTURE AND AMINO ACID SEQUENCE: Alpha-antitrypsin is considered a single chain polypeptide with one or more carbohydrate side chains. The biological site of action is present within a disulfide bond linkage. Twenty-four different variants of the molecule have been reported but only three have received any extensive biochemical analysis. Table I lists the amino acid and carbohydrate composition of variants M, S and Z. The composition for M and Z was reported by Miller and associates (1) while Roll and his colleagues reported the analysis of the S variant (11). The procedure used for amino acid analysis is accurate within one or two residues per molecular weight of the protein under investigation. Only six amino acids showed a variation in the number of residues per molecular weight and these variations were all confined to the S variant. Lysine and proline were 15% and 13% lower in the S variant compared to the other variants analyzed while glutamic acid, glycine and leucine were elevated 11%, 15% and 8% above their counterpart residues in the M and Z variants. While the same molecular weight was used to calculate the residues per mole for both the S and M variants, the two groups of investigators do not agree on the number of disulfide bonds present. Roll (11) found 3 half cystine residues in the M variant while Miller (1) reported 1.7 half cystine residues in the M variant. In all other respects, the two groups agree on the qualitative and quantitative amino acid composition of the M variant within the limits of the analytical procedure. Chan and Rees (2) report about a 50% increase in both arginine and glycine residues of the Z variant above the M variant.

TABLE I

COMPOSITION OF ALPHA-1-ANTITRYPSIN VARIANTS

Amino Acids	M	S	Z
Lysine	36	32.4	38.2
Histidine	11.8	11.1	12
Arginine	6.8	7.7	7.1
Aspartic Acid	40.8	42.4	41.1
Threonine	25.6	28	27.6
Serine	19.7	22	20.6
Glutamic Acid	47.1	51	45.3
Proline	7.7	16.7	19.2
Glycine	20.8	23.	99.6
Alanine	22.7	25.5	22.8
Half Cystine	1.8	3.5	1.7
Valine	22.2	22.8	23.0
Methionine	7.6	7.6	7.6
Isoleucine	16.3	15.1	15.9
Leucine	42.4	46.3	42.7
Tyrosine	5.2	5.0	5.6
Phenylalanine	23.9	22.7	22.5
Tryptophan	3.1	3.0	3.3
Carbohydrates			
N-Acetylglucosamine	11.6	16.4	10.9
Mannose	6.3	7.8	4.6
Galactose	5.2	6.7	3.3
Sialic Acid	7.6	7.1	5.6

Using tryptic peptide maps, Owen (12) reported the substitution of a neutral valine residue for a negatively charged glutamic acid residue on the S variant. Jeppsson (13) and Yoshida (14) found the substitution of a postively charged lysine residue for a negatively charged glutamic acid residue in the Z type variant. These and other unknown amino acid substitutions may promote structural changes which make it more susceptible to degradation and interrupt subcellular transport and secretion.

Koi and associates report that the N terminal group of rabbit alpha-antitrypsin is glutamic acid (3). The chemical composition of the product in rabbits shows slightly different quantities of protein residues especially methionine, glutamic acid and alanine based on a protein molecular weight of 58,000.

The carbohydrate moieties of this glycoprotein reside in one or more side chains. One of these chains blocks the N terminus of the peptide frame work. Chan and Rees (2) report the presence of four carbohydrate side chains consisting of two structural types. The first type consists of 4 residues of N-acetylglucosamine, 3 residues of mannose, 3 residues of galactose and 3 residues of sialic acid. The second side chain type consists of 3 residues of N-acetylglucosamine, 3 residues of mannose, two residues of galactose and 2 residues of sialic acid. These two types of oligosaccharide units are present in equal amounts in the M protein. The Z variant of the protein contains only three carbohydrate chains instead of four.

The actual quantitative carbohydrate content of alpha-antitrypsin is in dispute. Heimberger and associates found 12% of the total molecular weight of the glycoprotein to be carbohydrate (16) while Roll and his colleagues (11) report a 15-16% carbohydrate concentration. The quantity of each of the carbohydrate residues in the M, S and Z variants is shown in Table I. Crawford (17) also reports the presence of fucose and glucose moieties in the side chains but not enough to equal one molecular weight.

c) ELECTROPHORETIC MOBILITY: Routine human serum or plasma acid starch gel electrophoresis followed by crossed immunoelectrophoresis is the standard clinical method for detecting the presence, absence and typing of this protein (18). Twenty-four alleles or variants can be detected for alpha-antitrypsin using different types of electrophoresis. Table II indicates the different variant detected by diverse electrophoresis methods reported by Talamo (4).

Makino and Reed (6) have separated alpha-antitrypsin by electrophoresis through a 1% agarose gel using veronal buffer at pH = 8.6. Polyacrylamide gel techniques have been reported by Koi (3) and Miller (1). Roll (11) has used SDS polyacrylamide electrophoresis with this protein. In polyacrylamide gel the M variant has an electrophoretic mobility of 0.53 while the Z variant has an electrophoretic

TABLE II
DETECTION OF PROTEASE INHIBITOR ALLELES

METHOD	ALLELES
ACID STARCH GEL ELECTROPHORESIS	B, C, D, E, E_2, F, G, I, L, M, N, P, S, V, W, X, Y, Y_2
ANTIGEN-ANTIBODY CROSSED ELECTROPHORESIS	Z
ARGAROSE GEL ELECTROPHORESIS	F_2, $M_{BALDWIN}$, M_1, W_2
ANY QUANTITATIVE ELECTROPHORESIS	GENE FOR COMPLETE DEFICIENCY

mobility of 0.49. If the sialic acid moiety is removed from both variants their electrophoretic mobility slows to 0.48 but the direction of migration is reversed and goes toward the cathode (8). In the latter case treatment of the alpha-antitrypsin with neuraminidase will remove the sialic acid moeity but the biological activity remains.

Isoelectricfocusing is currently becoming fashionable for alpha-antitrypsin typing. Multiple bands will appear in a pH gradient between 4 and 6. Jeppsson and Laurell (8) using thin layer polyacrylamide, found a microheterogeneity with isoionic points between pH 4.5 and pH 4.9. They report that the charge of alpha-antitrypsin from two variants may be the same. Hercz and Barton (15) have found that only the isoproteins of alpha-antitrypsin stain with Schiff's reagent after electrofocusing in the pH range of 4.0-6.0. They also compare electrophoretic patterns of the glycoprotein for rabbit, goat, rhesus monkey, chicken and six human phenotypes on the same slide.

A two dimensional electrophoresis technique providing both high resolution and high specificity has been developed by Fagerhol and Laurell (19). Acid discontinuous starch gel (pH = 4.95) was used in the first dimension and crossed immunoelectrophoresis with rabbit anti-alpha-antitrypsin in the second dimension.

A paper electrophoretic technique was one of the first methods used to separate alpha-antitrypsin (20).

On routine serum electrophoresis, alpha-antitrypsin predominatly moves right behind the albumin and is usually associated with the alpha-1-globulin band. In some instances it has been reported as a "prealbumin" band (19).

d) ISOELECTRIC POINT: 4.5 to 4.9 because of its heterogeneity.

e) SOLUBILITY: Alpha-antitrypsin is soluble in water, physiological saline, 6 M urea, 3M sodium thiocyanate, tris buffer and sodium phosphate buffer. When 0.05-0.1M sodium chloride is added to either tris or phosphate buffers, however, the protein can be "salted out." It has been partitioned out of plasma by the use of organic solvents (33, 34) and loses its biological activity when in acid solutions below pH = 5.0 (33). It is insoluble in high concentrations of ammonium sulfate.

f) SPECIFIC GRAVITY: Miller and associates (1) report the partial specific volume of alpha-antitrypsin to be 0.711.

g) TISSUE CULTURE: Alpha-antitrypsin is produced in the hepatocytes of the liver (22) and has been synthesized in in vitro cultures according to Jeppsson and Laurell (8). In those people who have an alpha-antitrypsin deficiency, these liver cells still produce the protein but it never gets into the circulation. To our knowledge human hepatocytes have not been cultured for the purpose of producing this protein. It may be possible to do so. Hellung-Larsen and Frederiksen (43) have grown human liver cells in monolayer cultures using Eagle's Minimal Essential Medium supplemented with 10% fetal calf serum. Hepatocytes can be obtained by liver biopsy using sterile techniques. Takaoka and associates (23, 24) have shown that rat liver cells can grow in suspension cultures and do not

have a requirement to be attached to a surface. Using their new culture medium, designated DM-153, plus 10% fetal calf serum (23) their cultures of Japanese Albino Rat (JAR-1) strain liver cells grew 31 fold within two weeks and started to level off on the growth rate curve. The control hepatocytes, grown on Eagle's Minimal Essential Medium, leveled off on the growth curve after only five days. The doubling time for rat hepatocytes is about 32-48 hours based on analyzing the charts presented in the work of Takaoka (23).

The rate of alpha-antitrypsin production per cell has not been determined.

h) METHOD OF TISSUE CULTURE: Williams and associates (25) provide a very detailed procedure for the long term pure culture of rat hepatocytes. The cultures were still viable at the end of fourteen months but had shown signs of tumorigenicity when transplanted back into animals of the same species.

The procedure they used was the following:

1. At room temperature remove livers from ten decapitated rats about 10 days old. Yield will be about 3 grams of liver tissue.
2. The pooled livers are immediately minced with scissors in phosphate buffered saline.
3. The saline is drained and the minced tissue is passed through a tissue press containing 1 mm openings.
4. The tissue is collected in a 30 ml trypsinizing flask containing a 0.25% trypsin solution in phosphate buffered saline equivalent to 20 ml/gm of tissue added.
5. The trypsinizing flask is stirred magnetically for ten minutes and then the tissue is allowed to settle. Discard the supernatant containing red blood cells.
6. Repeat steps 4 and 5 but stir for 15 minutes.
7. Instead of allowing cells to settle, pour the suspension through a 230 mesh nylon screen and collect the filtrate in a 40 ml conical centrifugate tube.
8. Centrifuge at 600g at room temperature to pack the cells.
9. Aspirate the supernatant from the tube and discard.

10. Resuspend the pellet of cells in 10 ml of their modified Minimal Essential Medium by working the fluid and cells back and forth through a 10 ml pipette several times.
11. Pipette the cell suspension into a 75 cm^2 plastic tissue culture flask.
12. Incubate the flask at 36.5°C for 20 minutes in a 5% CO_2 and air environment.
13. Remove the medium, containing some cells, from the flask by pipette and put into a second flask which is also cultured.
14. The attached cells in the first flask are covered with 8 ml of their modified MEM medium.
15. Both culture flasks from steps 13 and 14 are returned to the incubator.
16. The flask from step 14 is removed from the incubator at the end of 20 minutes and subjected to steps 13 and 14.
17. All three culture flasks are returned to the incubator and 24 hours later the medium is replaced with the same mixture.
18. The medium is replaced on a weekly basis thereafter.

The special Eagle's Minimal Essential Medium modified by them contains vitamins and amino acid supplements as well as penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml). It can be obtained commerially from Grand Island Biological Company. This media is also supplemented with 10% fetal calf serum when added to cultures.

i) TECHNIQUE OF ASSAY: Several methods are available for the identification and assay of alpha-antitrypsin. Specific antibodies have been made against this protein for use in the Oucterlony test and for immunoelectrophoresis (27, 30). Fluorescein conjugated specific antibodies can be used for a qualitative test of alpha-antitrypsin presence (26). Single radial immunodiffusion test plates are commercially available (28, 29).

Probably the most common method for measuring alpha-antitrypsin function on a quantative basis is by measuring the ability of serum samples to inhibit the action of trypsin or chromogenic substrates such as benzoyl-p-nitroanilide (31) or on amino acid esters such as benzoyl-arginine ethyl ester. A spectrophotometric modification of this Trypsin Inhibitory Capacity (TIC) test read at 253 nm

has been reported by Homer and associates (32). Normally 1.1 mg of trypsin is inhibited per ml of serum using the chromogenic substrate benzoyl DL-arginine-p-nitroanilide (31).

EXPECTED SPACE IMPROVEMENT: No specific treatment now exists for severe alpha-antitrypsin deficiency. The use of whole plasma as replacement therapy would lead to immunological complications contravening the therapeutic benefit for the emphysema. Purified preparations of alpha-antitrypsin can be used as plasma substitutes to circumvent the immunological problem but the cost and time to produce pharmalogical quantities even for one person is prohibitive. The use of a continuous flow electrophoresis system in space to rapidly purify large quantities of alpha-antitrypsin from previously partially purified plasma may be the only approach available to make this a feasible pharmacological treatment procedure.

Any method in space or on earth that will increase the economic production of liver cells capable of producing this protein will establish a useful source for manufacturing. Until this is accomplished, the only source of supply is human plasma.

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NAME (GENERIC): Antihemophilic Factor (AHF), Factor VIII, Hemophilia A, Antihemophilic Globulin (AHG)

(PROPRIETARY): Profilate (Abbott Laboratories, Chicago, Illinois)
Factorate (Armour Laboratories, Kankakee, Illinois)
Koate (Cutter Laboratories, Berkeley, California)
Hemofil (Hyland, Costa, Mesa, California)
Actif VIII (Merieux, France)
Lyoc VIII (New York Blood Center)
Humafac (Parke-Davis, Detroit, Michigan)
AHF Concentrate (American Red Cross)
FI-O-Ta (Blomback, Sweden)
Feiba (Immuno Ag, Germany)

DISEASE TREATED: Hemophilia A, a hemorrhagic disease resulting from the absence of or deficiency of Factor VIII, one of thirteen known factors in plasma necessary for blood clotting.

PATIENTS: The number of hemophiliacs in the United States is questionable but at least 20,000 people. In 1973 The National Hemophilia Foundation (28) reported 100,000 hemophiliacs in the nation, 25,000 of whom are severely afflicted. Booz-Allen Company did a study for the National Heart and Lung Institute in 1971 in which they reported there were 24,499 American Hemophiliacs (30). In a subsequent 1976 Booz-Allen study they reported 13,287 people were afflicted with the disease (31). Drs. Barrow and Graham, prominent clinical investigators working with this disease, estimate 20,000 hemophiliacs in the United States (32). Our own calculations regarding the quantity of blood collected annually, the average size and frequency of dose required per patient and the estimated quantities of blood allocated to hemophilia treatment support the minimum 20,000 patient figure. A possible explanation of the large patient population discrepancy can be the inclusion of all hemophiliacs (i.e., those suffering from some form of blood coagulation deficiency) or the tabulation of only those who suffer from the more prevalent deficiencies, i.e., Factor VIII and Factor IX.

The disease primarily affects males and is transmitted through maternal genetic exchange. Through 1965 at least sixty women have been reported as confirmed Factor VIII hemophiliacs. Many patients currently under treatment appear to have developed

the disease mutationally (i.e., genetic patterns of forebears did not seem to relate to the development of the disease in the patient). This mutability plus the increased longevity of hemophiliacs into their reproductive years creates additional concern for pharmaceutical suppliers because of the potential increase in blood collection requirements to obtain Factor VIII as a product. According to the Director of Blood Collection at the American Red Cross Headquarters in Washington, D. C., his organization collected 4,600,000 pints of blood in the calendar year ending June 1976. He estimates that blood and plasma collected by pharmaceutical manufacturers handling blood products at least equaled the amount that the Red Cross collected and may be closer to double the quantity. In 1971 the National Heart and Lung Institute Survey of Blood Collection and Use indicated that over 9,000,000 pints of whole blood and 1,700,000 liters of plasma were collected by the blood service complex (an increase of 20% between 1967-1971). When it is considered that 20-25,000 hemophiliacs consumed 3.5-8.0 million pints of that blood and almost all of the plasma, any genetic shift to increase the number of patients may be a major strain on the ability to obtain blood.

SEVERITY ESTIMATE: Hemophilia can run the gamut from subclinical to clinical disability and death. Most patients are severely limited in mobility, both physiologically and in the freedom to travel away from home. Because the deficiency is usually detected in the first year or two of the patient's life, their whole life is affected. It is only within the last forty years that knowledge of the disease has advanced enough to initiate treatment by replacement therapy. As a result, hemophiliacs are starting to live longer. (The oldest is probably not 40 years old.)

TREATMENT REGIMEN AND METHOD: By necessity treatment must be individualized based on laboratory testing, patient body weight and the presence of AHF inhibitors. In general, each patient receives Factor VIII under two different circumstances, i.e., as a prophylactic measure to maintain blood coagulation capability at a specified level (usually 50%) and also as an emergency treatment to stop frank internal and external bleeding. The amount administered will vary in both situations and may be massive in quantity.

As a prophylactic measure most "mild" hemophiliacs receive an intravenous infusion of Factor VIII every third or fourth day. The spacing of the infusion depends on the half life of the Factor VIII being received and the amount of AHF

inhibitors present in the recipient. The "severe" patients have an AHF half life of about 8-13 hours while the more "moderate" or "mildly" affected patients report a Factor VIII half life of 2.9-3.6 days (32). The shorter the half life the more frequent the infusion. For severe hemophilics, Kasper and associates (33) have recommended a dosage of 250 units of Factor VIII per day in the morning for patients weighing less than 50 kg, and 500 units of Factor VIII for heavier patients. If bleeding episodes still occur too frequently, they recommend that the daily dose be progressively increased until a satisfactory degree of protection is obtained. Many older patients have developed antibodies to the commercial AHF VIII or its contaminating proteins (38). If this inhibitor is present in low titer, it may be overcome by increasing the prophylactic dose as much as 400 percent.

When there is frank internal or external bleeding, or surgery is anticipated, the amount of Factor VIII infused is elevated considerably. In the case of joint hemorrhages, 10 units/kg of body weight is administered at eight to twelve hour intervals for a period of one or more days depending on severity and patient response. This response may be measured by relief of pain, swelling and restriction of joint movement. Minor hemorrhages and massive hemorrhages in nonvital areas would be treated in the same way. Hemorrhages or overt bleeding near vital organs (neck, throat subperitoneal, etc) usually require 20 units/kg of body weight initially followed by 10 units/kg every eight hours for forty-eight hours or more. Prior to surgery the recommended dose is 40 units/kg followed by 20 units/kg every eight hours to maintain plasma Factor VIII levels to at least 30% (by laboratory testing) immediately prior to the next infusion. This infusion level should be maintained for at least ten days post-operatively. Following this dosage schedule an 80 kg adult hemophiliac would require the equivalent of 200 pints of plasma to survive an operation.

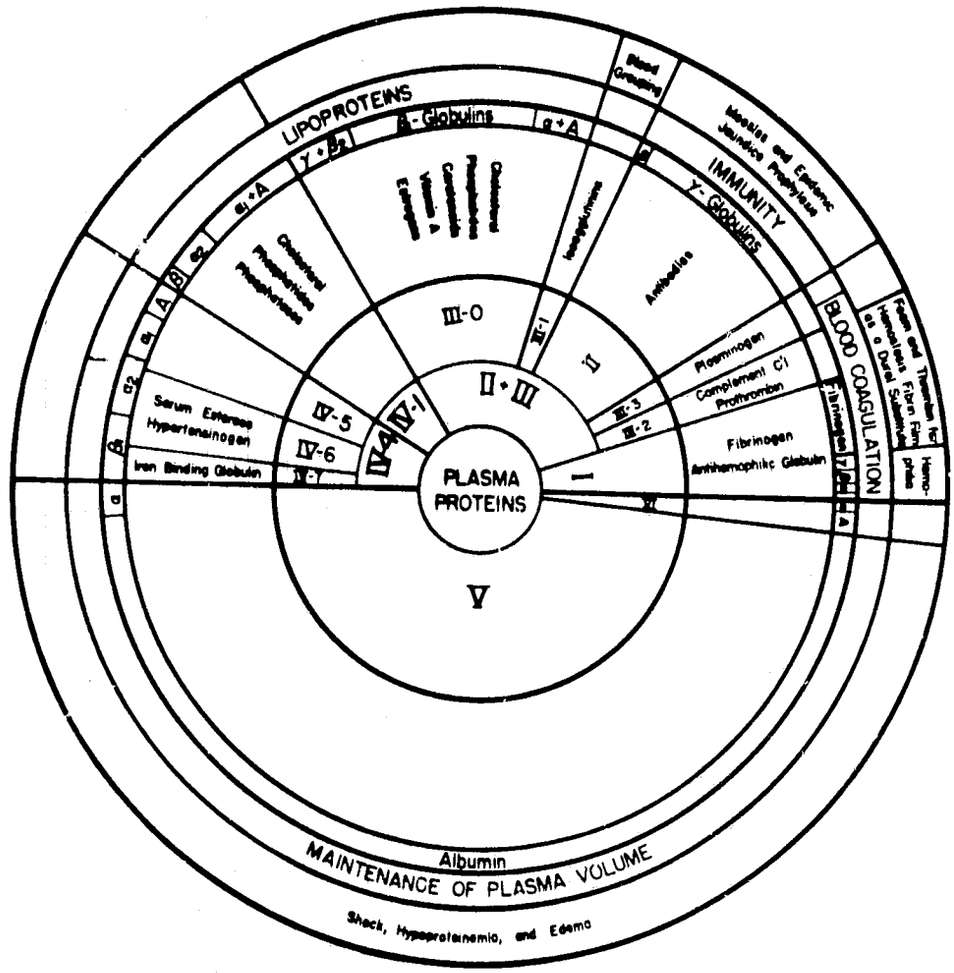
CURRENT GROUND STATUS: The increased longevity of hemophiliacs, allowing more of them to reproduce, and the evidence showing mutability of this gene indicate that the number of hemophiliacs may increase in the future. Just as a prophylactic measure, the approximately 20,000 people deficient in Factor VIII currently consume the AHF plasma concentrate from 3.5-8.0 million pints of blood collected annually through 5,400 blood centers in the United States. The only known source of AHF Factor VIII is the blood plasma of other humans. While AHF Factor VIII is present

in the blood of other animals, it is not used for replacement therapy because of the consequences of developing severe antibody reactions to foreign contaminating proteins after several weeks of injections. If the molecular structure of AHF in man and animals were known to be identical, then replacement therapy by substitution would be possible assuming isolation and purification.

The actual source of AHF synthesis is not known. For more than twenty years the cellular sites of production have been sought by a variety of experimental approaches. This field of activity is summarized by Barrow and Graham (32). Experiments requiring organ removal, chemical intoxication, radiation and hemorrhaging suggest that the cells of the pancreas, spleen, liver, and reticuloendothelial system are not solely responsible for the synthesis of Factor VIII. Other experimental data seem to contradict these conclusions. The difference in results may reflect better assays and the larger amount of experimental evidence in the later experiments. Dodds (34) perfused isolated rabbit livers, spleens, lungs and kidneys and recovered Factor VIII activity from all organs. The experiment of Marchioro and his associates (35) transplanting homologous hemophiliac livers into normal dogs did not result in a complete loss of AHF production and indicated that extrahepatic sites play a major role in production of this protein.

b) STARTING MATERIAL AND PRODUCTION METHOD: Factor VIII at the present time can only be obtained from the plasma of other humans. Approximately 5,400 blood collection centers in the United States are engaged in the collection of Factor VIII containing plasma using phlebotomy and plasmapheresis techniques. Once this plasma is collected, it must be adequately mixed with an anticoagulant and rapidly chilled or frozen. Beyond this point several alternate methods are employed for extraction of Factor VIII.

The major breakthrough in AHF separation came during World War II when Cohn (20) developed a plasma fractionation procedure using ethyl alcohol, low temperature and carefully controlled pH and ionic strength. Cohn's Fraction I of the plasma is insoluble when frozen and rethawed at just about freexing (4°C). It contains much of the Factor VIII activity and practically all of the fibrinogen. It is also contaminated with albumins, α_2 and β globulins, plasminogen and other plasma proteins (see Figure I). This Fraction I is more commonly known as cryoprecipitate and is removed from the plasma by centrifugation or gravity drainage.



Plasma Proteins - Their Natural Functions and Clinical Uses and Separation into Fractions. (Revised by L. E. Strong from Figure 1, in Cohn, E. J., "Blood Proteins and Their Therapeutic Value," **Science 101:54, 1945.**)

FIGURE 1. COHN PLASMA FRACTIONS

To remove the other proteins in the cryoprecipitate, additional precipitation procedures are conducted. When the cryoprecipitate is resuspended in a solution containing 11% ether at 0°C , the fibrinogen and AHF Factor VIII are selectively precipitated (21). This material is centrifuged, separated from the supernatant, lyophilized and sold as an intermediate potency Factor VIII.

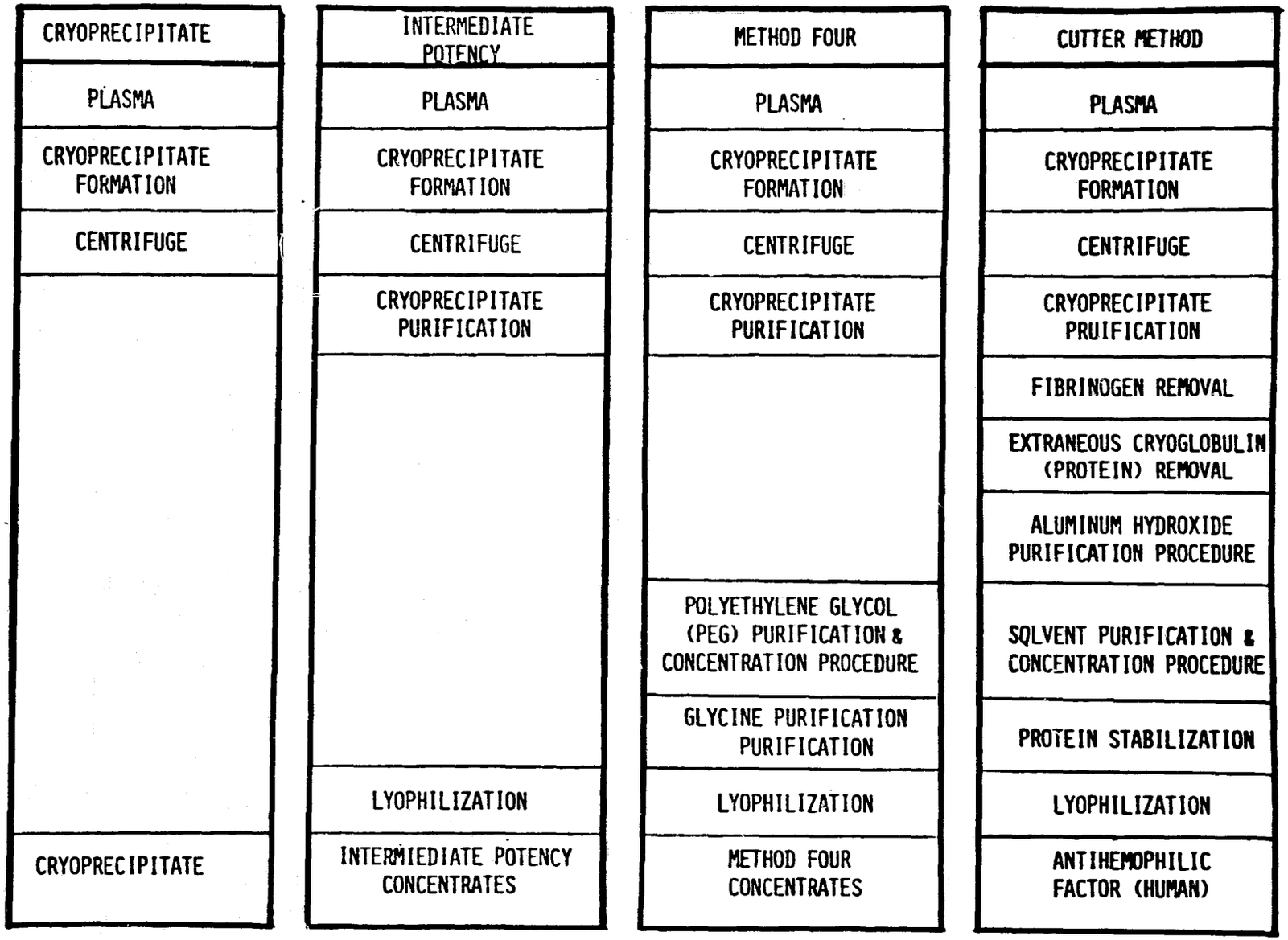
A Factor VIII fraction of high potency (METHOD IV) has been produced commercially and has come into wide spread clinical use in America (22, 23). Using plasma cryoprecipitate as the starting material, it is subjected to "salting-out" precipitation using polyethylene glycol and glycine. The American Red Cross has also developed a high purity AHF from cryoprecipitate by treating that material with polyethylene glycol and ethanol (24).

A chart of comparative processing techniques to obtain AHF from plasma is shown in Figure II. All of the processing of the plasma from immediately after collection to lyophilization must be done in the cold (0°-4°C) to prevent enzyme destruction of the AHF. Sterile filtration using banks of asbestos sheets several feet thick is carried out on the final solution immediately prior to lyophilization.

Almost all current methods have fibrinogen as a contaminant in the marketed product. During analysis of one commercial AHF product, our laboratory determined that it contained at least 95% contaminanting protein, probably fibrinogen. Other contaminating products, depending on manufacturer, can include polyethylene glycol and some unidentified stabilizing agents. The literature indicates that Factor VIII can be removed from fibrinogen by free film electrophoresis (25) by selective adsorption of fibrinogen with diatomaceous earth (26) by ion exchange chromotography (27) or by filtration through argarose gel (3). Apparently none of these procedures is employed commercially because of the resultant relatively high losses of the AHF.

Since the site of AHF synthesis has not been identified, tissue culture methods for producing this protein are still far into the future.

FIGURE 2 COMPARATIVE PROCEDURES IN AHF CONCENTRATE PROCESSING



c) YIELD: The yield of Factor VIII will vary depending on the separation technique and starting material employed but in general it will not exceed 83% of the original starting value (29, 37). In most cases the commercial product will represent only a 25% yield.

d) PURITY: Almost all current commercial methods have fibrinogen as a contaminant in the marketed product. Polyethylene glycol, heparin, a stabilizing agent and other unknown protein materials may also be found in the AHF Factor VIII depending on the brand of the pharmaceutical product obtained. The major problem to the recipient is the possible presence of Hepatitis B virus. The Department of Health, Education and Welfare estimated 17,000 cases of post-transfusion hepatitis in 1975 resulting in at least 850 deaths (28). Of almost equal concern is the presence of the other impurities listed above because they may produce antigen-antibody reactions or develop inhibitors.

e) COST/DOSE (to patient) AND AVAILABILITY: The American Red Cross currently charges $0.11/unit for cryoprecipitate and $0.14/unit for the partially purified Factor VIII. The commercial manufacturers charge higher prices for their products. The National Hemophilia Foundation reported that the cost to a patient of a 250 unit bottle of Factor VIII can range from $3.50 to $50.00 across the country (28). This differential is the result of varying governmental and insurance subsidies for the product. States like Missouri and Ohio pay for a substantial portion of the pharmaceuticals required by hemophiliacs. There are seven American manufacturers of Factor VIII and three in Europe, all selling to the American market. These companies are Abbott, Armour, Cutter, Hyland, Parke-Davis, American Red Cross, New York Blood Center, Merieux (France), Blomback (Sweden) and Immuno Ag (Germany). In addition to these large scale pharmaceutical manufacturers, many hospital blood banks also process blood for hemophiliac needs.

Factor VIII is only available by prescription and in most cases must be administered in a hospital or out patient clinic affiliated with a hospital. Therefore almost all sales are directly to hospitals or physicians treating this type of patient. In the few instances, about 10% of the patients, where the attending physician has trained the older hemophiliac to treat himself, he may bring the Factor VIII home from the hospital for self administration as needed. The local

pharmacies do not ordinarily carry this product. A survey of the St. Louis Metropoliton area (approximate population two million) indicated only one pharmacist stocking Factor VIII. He would only sell the AHF in case quantities.

f) STORAGE: Factor VIII, when lyophilized, must be stored under refrigeration at a temperature of 2°-8°C. Storage of lyophilized powder at room temperature for short periods of up to one month can be tolerated without loss of activity. Reconstituted Factor VIII (i.e. put back into solution, should not be refrigerated and should be used within three hours after reconstitution. Factor VIII in cryoprecipitate must be stored at -30°C or below to prevent deterioration (36).

TECHNICAL DATA

a) MOLECULAR WEIGHT: Accurate estimates of the molecular weight of Factor VIII have been hampered by difficulties in purifying it. The literature reports a molecular weight range from 25,000 (1) to greater than 2,000,000 (23). It is thought that the higher molecular weights may actually be aggregates of smaller weight moieties. The Factor VIII-like activity from the kidney is reported to have a molecular weight of about 25,000 (4) and may be bound to a "carrier" molecule for transport throughout the body. Anderson (5) and Weiss (2) both broke the 2,000,000 dalton AHF molecules into 195,000 and 194,000 daltons respectively, using electrophoresis and salting out techniques. Owen and Wagner fractionated canine plasma into a fully active fragment of about 100,000 daltons (6). The concept of aggregation of smaller fragments or the bonding of an active fragment to a high weight carrier protein has been given credence by the reassociation work of Cooper and his colleagues (7).

b) MOLECULAR STRUCTURE AND AMINO ACID SEQUENCE: It is generally assumed that Factor VIII is a protein but this has been questioned by a number of investigators. Veder (8) and Johnson (9) did not find a typical protein absorption peak at 280 nanometers in their highly purified AHF material. Absorbance was observed at 210 and 230 nanometers, however. Hershgold and associates (10) analyzed a human AHF fraction of high purity and found it to contain 75% amino acids, 10% hexose and 11% lipids. Marchesi (11) found only 5% carbohydrates, 5% lipids and the balance protein in his human plasma AHF preparation. Bovine Factor VIII contains 20% Carbohydrate with the protein but no lipid (12). The latest biochemical data therefore

seem to imply at the least that human plasma Factor VIII is probably a glycoprotein, not a simple polypeptide. Because of the uncertainty of the molecular size of this protein, the amino acid composition and sequence has not been determined.

c) ELECTROPHORETIC MOBILITY: Antihemophilic Factor VIII has been subjected to a variety of electrophoresis techniques. Each technique has influenced the electrophoretic migration of the protein and as a result the active fraction has appeared in different locations in the plasma protein fractionation pattern. Using paper electrophoresis, AHF was reported to migrate in the β_1 or β_2 globulin region (13). Lewis and associates (14) found Factor VIII activity of plasma in the prealbumin and α_1-α_2 regions using continuous flow electrophoresis on a paper curtain. Kass (3) demonstrated that human Factor VIII migrated in the γ region when electrophoresed on polyacrylamide gel. Starch-block electrophoresis presented Factor VIII in the α_2 globulin region (15). Using a continuous free flow electrophoretic technique with Tris-Barbital buffer, the McDonnell Douglas Aerospace Medical Department separated AHF in the post albumin region. The AHF had an electrophoretic mobility of 7.8×10^{-5} cm^2/volt-sec.

d) ISOELECTRIC POINT: This value has not been determined to our knowledge.

e) SOLUBILITY: AHF is freely soluble in water and physiological saline. The lyophilized product is currently supplied commercially for reconstitution with distilled water at a solubility of 25-50 mg cryoprecipitate/liter and can be resuspended as a 12% solution at body temperature (29). Factor VIII precipitates readily with salts, organic solvents, glycine, ether, diethyl-ether, alcohol, tannic acid, polyethylene glycol and citrate buffers. The standard method of initial separation from other plasma proteins is by cryoprecipitation. The resulting cold insoluble globulins do contain the bulk of the Factor VIII activity and fibrinogen.

Factor VIII is very sensitive to pH and temperature changes. At the pH of venous blood, 7.2, more than 90% of the activity persisted for 7 days when the material was kept at 4°C. If the material was stored at 28° or 37°C under the same conditions, there was a 50% loss in AHF activity within 24 hours. Elevating the temperature to 45°C caused 50% loss of activity in 30 minutes. At 55°C or above

the material was denatured almost immediately. When plasma was maintained at body temperature for 30 minutes, activity was lost completely if the pH exceeded the pH extremes of 4 and 10. At a pH range of 5 and 9 there was a 50% loss of activity under the same time and temperature conditions.

f) SPECIFIC GRAVITY: Because the cells producing AHF have not been determined, their specific gravity is unknown at this time.

g) TISSUE CULTURE: The site of Factor VIII synthesis is unknown at this time. Because a number of abdominal organs and even the isolated hind limb (16) have been reported to produce this product, replacement has obviously concentrated on organ transplantation rather than cultures of specific cells.

h) METHOD OF TISSUE CULTURE: See Section g.

i) TECHNIQUE OF ASSAY: Estimates of the amount of Factor VIII in plasma have been based on three assay procedures. The oldest procedure involves measuring the improvement of the impaired utilization of prothrombin in hemophilic whole blood after the addition of varying amounts of plasma being tested (17). The estimate is made by comparing the effectiveness of the test plasma with a standard plasma in bringing the prothrombin concentration of a hemophilic plasma to some pre-selected value under standard conditions, e.g. 50% in 30 minutes at 25°C and pH 7.0. Another assay of Factor VIII is based on the observation that the prolonged "partial thromboplastin" clotting time (PPT) of hemophilic plasma can be reduced by addition of small amounts of normal plasma (18). The effectiveness of a test plasma can be compared with that of a normal plasma in reducing the prolonged clotting time and the effectiveness expressed as a percent of the normal. The third method (19), the thromboplastin generation test (TGT) measures the amount of "thromboplastin" generated in a clotting system in which the amount of Factor VIII is the limiting element.

EXPECTED SPACE IMPROVEMENT: Electrophoretic separation in space can greatly increase both the yield and purity of Factor VIII. The improved fractionation procedure will also allow more utilization of the same plasma protein components so that one pint of plasma can serve more than two functions. This increased utilization and yield can reduce the large quantities of blood currently being collected, remove some of the patient hardships resulting from contamination and may ultimately bring the cost down through expanded opportunities to profit on the same material.

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NAME (GENERIC): Epidermal Growth Factor (EGF)
(PROPRIETARY): CR-EGF (Collaborative Research Company, Waltham, Mass.)

DISEASE TREATED: Skin Replacement for third degree burns and ulcerous lesions
Wound healing after injury or surgery

COMMERCIAL USE: Tissue Culture Production
Stimulate Cell Proliferation and Protein Synthesis
Reduce Animal Serum Requirements

PATIENTS: According to the National Fire Protection Association, just under 11,000 persons died in fires within the United States and Canada during 1977. Another 139,500 persons were seriously injured by fire over the same period of time. While some of this group probably succumbed to, or were hospitalized for, asphyxiation as the result of smoke inhalation, many of these patients received third degree burns. No statistics are available enumerating the actual number of people in this category. Discussion with directors of several hospital burn units throughout the country has resulted in a consensus of approximately 14,000 patients annually receiving treatment for third degree burns over at least five percent of their body.

The number of people that may benefit from using EGF for wound healing is unknown but is believed to be in the hundreds of thousands annually in the United States alone. It could be used for the rapid healing of abrasions and lacerations as well as the epidermal repair of surgical procedures.

SEVERITY ESTIMATE: The medical procedures to which this product can be applied range from the cosmetic problems of simple abrasions to the terminal condition of severely infected third degree burns over most of the body.

TREATMENT REGIMEN: This product has been suggested for inclusion in this study by more than one company contacted. To our knowledge there is no currently available open literature describing the use of EGF for the treatment of any of the medical conditions listed above. Extensive pharmacological research with this material, however, is being conducted by two drug companies (one American

and one foreign). The companies wish to keep this information proprietary for the present. In order to provide a rough order of magnitude dosage, for comparison purposes with other products, we have chosen a value of 20 nanograms/cm^2 which causes epidermal cells in tissue culture to proliferate and spread into a fibroblast network (17).

TREATMENT METHOD: A third degree burn is one in which the skin, and possibly the underlying tissue, is damaged beyond normal repair. While this skin is missing, body fluid will constantly discharge through the exposed surface area making it highly receptive for the growth of micro-organisms. Depending on the extent of tissue damage and control of both the infection and fluid loss, the patient may not survive in spite of heroic measures with protein-fluid replacement and massive antibiotic therapy. The faster the protective barrier of skin is restored to its original condition, the less risk of exposure to the patient.

After the damaged skin has been surgically removed (debrided) back to the non-injured tissue, there are two methods for its resurfacing. The first method is to allow the edges of the intact skin surrounding the damaged area to proliferate and gradually fill in the wound. In those situations where the damaged area is extensive, the second method is to replace the skin through dermal grafts taken from other locations on the patient, if physiologically permissible, or from other people. When skin from another person is grafted, there is a strong probability that the recipient's body will recognize the tissue as "foreign" and reject it by sloughing it off within one to six weeks. During this period of time, the edges of the damaged skin will begin to proliferate and gradually recover the area through encroachment. By using EGF, the cells on the edge of the injured area will be stimulated to proliferate very rapidly to cover the area. When the burn areas are extensive, autologous cells soaked in EGF can be sprinkled into the denuded area where they should attach and rapidly proliferate toward the encroaching edge.

COMMERCIAL USAGE REQUIREMENTS AND METHODS: Epidermal Growth Factor appears to have many uses for the production of cells in tissue culture. The addition of EGF to tissue cultures of human epidermis has resulted in lengthening the life of the culture from 50 to 150 generations above controls (19). Chen and associates

(18) have confirmed that the use of EGF can drastically reduce the need for fetal calf serum as a nutrient in tissue culture (3). Most cell culture media are usually mixed with 5-10% fetal calf serum. Only 0.02% of the serum is required, however, in the presence of EGF at a concentration of 1-2 nanograms/ml. Many highly differentiated vertebrate cells need to adhere to some surface for continued growth. A protein, known as large external transformation sensitive (LETS) protein is reputed to be responsible for this attachment. Most transformed cells and tumorigenic cells consistently show a reduction or absence of this protein. Addition of EGF at a concentration of 1 nanogram/ml to tissue cultures was able to restore these LETS protein networks (18).

Approximately twelve companies collect and process fetal calf, calf and horse serum in the United States. Annual production amounts to about 150,000 liters of serum with fetal calf serum accounting for half of the supply. Each calf fetus or "slunk" must be vacuum bled through intracardiac puncture within several minutes after the mother is slaughtered. Usually only 1.0-1.5 liters of serum will result from each fetus. Because of the currently high cost of beef production, very few farmers are shipping pregnant cattle to slaughter. This has resulted in a shortage of the serum and an inflated cost of sixty dollars per liter. If the current clinical trials with EGF verify its pharmacological potential, large quantities of fetal calf serum will be required to culture the cells that produce EGF. This need will probably not be met unless the price of the serum increases several fold.

About 15,000 liters of horse serum are produced annually for the veterinary medicine market. The current cost of the serum is forty-five dollars per liter. While each horse can supply up to ten liters of the serum several times a year, freedom from certain types of diseases, i.e., equine encephalitic anemia, necessitate that herds be kept for this purpose.

Use of epidermal growth factor to supplement a reduction in fetal calf serum requirements in tissue culture would stretch the supply of serum about five hundred times. Even at the current cost of $495/mg for EGF, use of 2 nanograms/ml of the product with 0.02% fetal calf serum would tremendously reduce the cost of tissue culture.

CURRENT GROUND STATUS: Epidermal Growth Factor was first isolated from the submaxillary glands of mice over fifteen years ago by Cohen. Recently it has also been isolated from human urine. In the intervening time since its discovery, the protein has been purified and its amino acid sequence determined. Because of its very low concentrations in animal and human plasma it is very difficult to extract in purified form (the process usually taking 7-10 days). This has resulted in only research quantities of the protein being available. As a result, very little investigation of its clinical potential has been accomplished. Two pharmaceutical companies are conducting research in the area of burn treatment but they wish to keep the status of their work proprietary at this time.

Work is needed in the areas of both large scale purification of the material and the development of large tissue cultures as a source of supply. When both tasks are accomplished, EGF will stand a good chance of becoming a viable commercial product for tissue culture media supplementation. As information becomes available concerning its use with burn patients, a pharmaceutical market should develop in this area.

Epidermal Growth Factor is unique for both the tissue culture research - production market and the burn treatment market. Even at the current cost of $495/mg, EGF can reduce the fetal calf serum cost for each 100 liters of culture media change from approximately $600 to $61.20 (sixty dollars for the required epidermal growth factor and one dollar twenty cents for the fetal calf serum). As EGF can be produced in higher quantities at cheaper cost the price of EGF should go down. In the field of burn medicine, any process or product that would induce rapid healing of the burn site while reducing the amount of scar tissue formation would be immediately welcomed.

b) STARTING MATERIAL AND PRODUCTION METHOD: Two methods are currently used for the isolation and purification of EGF depending on the source of the material. Both use relatively involved protocols taking about 7-10 days for execution but result in a final product that can be used for amino acid sequence analysis. Lyophilization after each major extraction step allows the material to be held for periods of time during the total processing procedure rather than requiring a continuous operation.

Savage and Cohen have reported a new procedure (1) for the rapid isolation and purification of murine EGF from submaxillary glands. This procedure is based on the observation that, at low pH, polyacrylamide gel will selectively adsorb EGF from crude homogenates of the submaxillary glands. Starting with 30 gms of frozen submaxillary glands collected from 150 male mice, they used the following procedure to obtain a 19 mg yield of EGF:

1. Homogenize thawed submaxillary glands with 118 ml of cold 0.05M acetic acid in a blender at 4°C for 3 minutes.
   NOTE: pH must be adjusted to 4.5 immediately upon completion of homogenization or enzymes in the solution will remove two terminal amino acid residues.
2. Freeze homogenate in a dry ice-alcohol bath.
3. Thaw and centrifuge at 100,000g for 30 minutes.
4. Collect supernatant by decanting through glass wool to remove floating fat particles.
5. Resuspend the centrifuged pellet in 74 ml of 0.0005M acetic acid followed by centrifugation at 100,000g for 30 minutes.
6. Repeat steps 4 and 5.
7. After the third supernatant is decanted through the glass wool, all three supernatant materials are combined and lyophilized. The pellet is discarded.
8. Resuspend the lyophilized powder from step 7 in 7 ml of cold 1N HCl to rapidly lower pH.
9. Dilute the solution in step 8 with 18M of 0.05N HCl adjusting the final pH to 1.5.
10. Centrifuge at 100,000g for 30 minutes at 4°C.
11. Collect the supernatant.
12. Resuspend the resultant pellet in 3 ml of 0.05N HCl and repeat step 10.
13. Combine supernatants and chromatograph on an acid Bio-Gel P-10 column at 5°C.
    NOTE: 5 x 90 cm column was packed with Bio-Gel P-10 (100-200 mesh) and equilibrated with an HCl-NaCl buffer (0.05N HCl containing 0.15M NaCl) at 5°C. Flow was set at 45-50 ml/hr at a pressure head of 36 cm.
14. Monitor elution at 280 nm.
15. Collect 25 ml fractions over range of 2825-3475 ml of column eluate.

16. Neutralize pooled collected fractions to a pH between 5 and 7 with 1N NaOH.
17. Concentrate solution to approximately 10 ml using pressure ultrafiltration with an Amicon UM 2 membrane.
18. Add 250 ml of 0.02M ammonium acetate (ph = 5.6) to concentrate.
19. After mixing, repeat step 17.
20. Apply concentrate to a DEAE absorption column.
    NOTE: 1.5 x 20 cm column was packed with DEAE-cellulose and equilibrated with 0.02M ammonium acetate (pH = 5.6) at 5°C. Flow was maintained at 12 ml/hr.
21. Wash column with two column volumes of 0.02M ammonium acetate.
22. Elute EGF column using a gradient solution of ammonium acetate.
    NOTE: Gradient is formed by allowing 0.2M ammonium acetate buffer to flow into a 125 ml constant volume mixing chamber containing 0.02M buffer. Both buffers were set at pH = 5.6.
23. Monitor the eluate at 280 nm.
24. Collect fractions of major peak (usually fractions 15-25 when 6 ml/ fractions are collected).
25. Pool fractions containing EGF and lyophilize.
26. Dissolve lyophilized powder in 10 ml of 0.05M acetic acid.
27. Relyophilize solution and store desiccated at 5°C.

Human EGF has been isolated from urine of pregnant females but is not unique to either pregnanacy or the female gender. Fifteen liters of human urine were passed through a benzoic acid column to remove any protein. The column was then washed with acetone and vacuum dried to remove any traces of water. The resulting 10 grams of the acetone/benzoic acid powder contained 800 mg of urinary proteins. Starting with this acetone/benzoic acid powder, Cohen and Carpenter (3) have developed the following procedure for isolating and purifying 200-300 micrograms of EGF from the adsorbed protein.

1. Suspend 10 gms of a benzoic acid/acetone powder of urinary proteins in 40 ml of water and adjust solution to pH = 9 using 1M NaOH.
2. Centrifuge mixture at 18,000 RPM for 10 minutes in a 4°C environment.
3. Collect supernatant.

4. Resuspend residue in 50 ml of cold water and repeat step 2.
5. Combine supernatants from steps 3 and 4 and lyophilize.
6. Redissolve lyophilized powder in 18 ml of water and centrifuge as in step 2.
7. Add supernatant to a Bio-Gel P-10 chromatography column.
   NOTE: The 2.5 x 90 cm column was packed with Bio-Gel P-10 (100-200 mesh) and equilibrated with 0.01M ammonium acetate at 4°C. The flow rate was 2.8 ml/hr and 15 ml fractions were collected.
8. Elute with 0.01M ammonium acetate and monitor at 280 nm.
9. Fractions 22-26 are collected, combined and lyophilized.
10. Redissolve the lyophilized product derived from step 9 in 3 ml of water and apply to a Sephadex G-50 column.
    NOTE: This 1.5 x 90 cm column was equilibrated with 0.01M ammonium acetate at 4°C. The flow rate was 11 ml/hr and 5 ml fractions were collected.
11. Elute with 0.01M ammonium acetate and monitor at 280 nm.
12. Fractions 20-25 are collected combined and lyophilized.
13. Redissolve the lyophilized product derived from step 12 in 11 ml of 0.03M formic acid (pH = 3.0) and apply to a chromatography column of DE-52 cellulose.
    NOTE: This 0.9 x 8.5 cm column was equilibrated with 0.3 ammonium formate buffer at pH = 3.0.
14. Elute with 8 column volumes of the same buffer.
15. Lyophilize the eluate.
16. Dissolve product from step 15 in 16 ml of 0.04M acetic acid (pH = 4.0) and apply to ion exchange column packed with CM-52 cellulose.
    NOTE: This 1.5 x 10 cm column was equilibrated with 0.04M ammonium acetate at pH = 4.0.
17. Wash column with 50 ml of the buffer.
18. Elute the column with a gradient solution of ammonium acetate.
    NOTE: The gradient was prepared by allowing 2.0M ammonium acetate buffer to flow into a 125 ml constant volume mixing chamber filled with 0.04M ammonium acetate solution.
19. Monitor the elution at 280 nm.
20. Using 5 ml sized fractions, EGF can be found in fractions 38-47.

21. Collect, combine and lyophilize fractions 38-47.
22. Redissolve the lyophilized product of step 21 in 5 ml of 0.02M ammonium acetate buffer with a pH = 5.6.
23. Apply to an ion exchange column containing DE-52 equilibrated with the same buffer. This column was the same size as step 13.
24. Wash column with 4 column volumes of the buffer.
25. Elute with an ammonium acetate gradient buffer.
    NOTE: This gradient was established by adding 0.2M ammonium acetate to a 65 ml constant volume mixing chamber containing 0.2M ammonium acetate.
26. Monitor at 280 nm.
27. Collect 2.5 ml fractions surrounding second major peak (usually fractions 26 through 30).
28. After fraction 38 is eluted from the chamber a second elution gradient is established by permitting 1.0M ammonium acetate, pH = 5.6, to flow into the constant volume mixing chamber.
29. Monitor at 280 nm.
30. A third peak containing EGF will appear in fractions 43 through 48.
31. Collect, combine and lyophilize fractions 43-48.

c) YIELD: The yield of EGF from male mice submaxillary glands by a gel exclusion chromatography procedure (1) was estimated to be approximately 0.5% of the submaxillary gland protein (2). In a typical preparation, the approximate total wet weight of 24 gms of submaxillary glands collected from 150 adult male mice yielded 5.7 mg of EGF with a recovery of about 20% of the original physiological activity. Approximately 700 microgram equivalents of human EGF (3) were present in 10 gms of a benzoic acid/acetone powder extract of urinary proteins, obtained by pooling 15 liters of human pregnant female urine. When this starting material was subjected to the EGF isolation procedure described by Cohen and Carpenter, the yield was about 20% of the initial concentration.

d) PURITY: The EGF isolated from mice submaxillary glands appears to be pure by several different criteria. Examination by paper electrophoresis with buffers of various pH values, SDS disc-gel electrophoresis and paper chromatography in a number of solvents presents a single locus when stained with appropriate protein indicators (2). It is antigenic resulting in only one precipitin band when

cellulose acetate immunoelectrophoresed against its antiserum. The immunological identity of EGF has been confirmed by cross reaction with antiserum generated against this material purified by other independent investigators (4).

Human EGF isolated from urine will react with mouse EGF antibody even though it has a different molecular weight and isoelectric point (3). This material also presents a single band with disc gel electrophoresis and also when subjected to an ultracentrifugal field.

As currently supplied for research use, EGF is stabilized with protein supplements (4). The supplements have not been identified by the supplier but they may be the high molecular weight binding protein described by Cohen and Taylor (2). If this is the case, it can readily be separated by any of the following methods: a) ion exchange columns, b) gel filtration in buffers below pH = 5.0 and above pH = 8.0, and c) with isoelectric focusing using low pH range ampholyte solutions (2). These authors suggest about a 16% yield of the mouse EGF from the EGF-binding protein complex.

e) COST/DOSE (TO PATIENTS) AND AVAILABILITY: EGF is currently available only for non-human research purposes. The only commercial supplier known is Collaborative Research, Inc., Waltham, Massachusetts (4). Their lyophilized, protein supplement stabilized mouse EGF is supplied in 100 microgram quantities for $60.00 and 1 milligram quantities for $495.00.

f) STORAGE: The water soluble lyophilized EGF supplied by Collaborative Research, Inc. is stable at room temperature. When reconstituted, the supplier recommends storage at -20°C. Cohen and Taylor (2) report that EGF biological activity was stable to boiling water but was destroyed by heating in dilute alkali and dilute acid. Biological activity was destroyed by incubation with chymotrypsin or a bacterial protease but only partially lost after incubation with trypsin.

TECHNICAL DATA

a) MOLECULAR WEIGHT: Material isolated, purified and characterized from mouse submaxillary glands has a molecular weight of 6045 based on a 53 residue polypeptide (12). Prior physical chemistry measurements had suggested a molecular

weight range of 6,166 to 7,000 depending on the method employed (2). The EGF isolated from human urine has a lower molecular weight of about 5,400 based on 49 amino acid residues (3).

b) MOLECULAR STRUCTURE AND AMINO ACID SEQUENCE: Epidermal Growth Factor is a single chain polypeptide with a nonhelical secondary structure. The chemical composition of mouse (12) and human (3) EGF is listed in Table I. The amino acid compositions of the two polypeptides differ in both the total number, types and quantities of amino acid residues present but there is much similarity. Human EGF has 49 residues as compared to 53 residues found in mouse EGF. Lysine and alanine are present in human EGF but not in mouse EGF. Threonine is lacking in human EGF but present in mouse EGF. Seven other amino acid residues differ in quantities found within these polypeptides. No neutral sugars and hexosamines have been identified with the molecule. Three disulfide bonds are present in both polypeptides and are all required for biological activity. The locations of the disulfide bonds has been determined for mouse EGF (13) and agrees with that molecule's amino acid sequence reported by Savage, Inagami and Cohen (12). This sequence and bonding is shown in Figure 1. The carboxyl terminus is arginine and the amino terminus is asparagine. To our knowledge the amino acid sequence of the human polypeptide has not been reported in the literature.

The EGF binding protein usually found with epidermal growth factor has a molecular weight of about 74,000. It is an arginine esterase. Since EGF possesses an arginine residue at the carboxyl terminus, Cohen and Taylor suggest that this growth factor may be generated from a percusor protein by the possible proteolytic action of the EGF-binding esterase.

c) ELECTROPHORETIC MOBILITY: This material readily moves in an electrophoretic field as observed with several different techniques including paper, gel, SDS-gel, and immuno-electrophoretic and isoelectric focusing procedures. The actual electrophoretic mobility will vary with each procedure and was not reported using SDS-page gel electrophoresis. A very fast electrophoretic mobility has been demonstrated for mouse EGF in relation to albumin, chymotrypsinogen and cytochrome c controls (4). The molecular weight of EGF would give this value credibility because it falls on the molecular weight versus SDS-gel electrophoretic

Table I
Amino Acid Composition of Epidermal Growth Factor

	Residues/Mole	
Amino Acid	Mouse	Human
Lysine	0	3
Histidine	1	2
Arginine	4	2
Aspartic	7	7
Threonine	2	0
Serine	6	3
Glutamine	3	5
Proline	2	2
Glycine	6	5
Alanine	0	2
Cysteine	6	6
Valine	2	2
Methionine	1	1
Isoleucine	2	2
Leucine	4	4
Tyrosine	5	2
Tryptophan	2	1

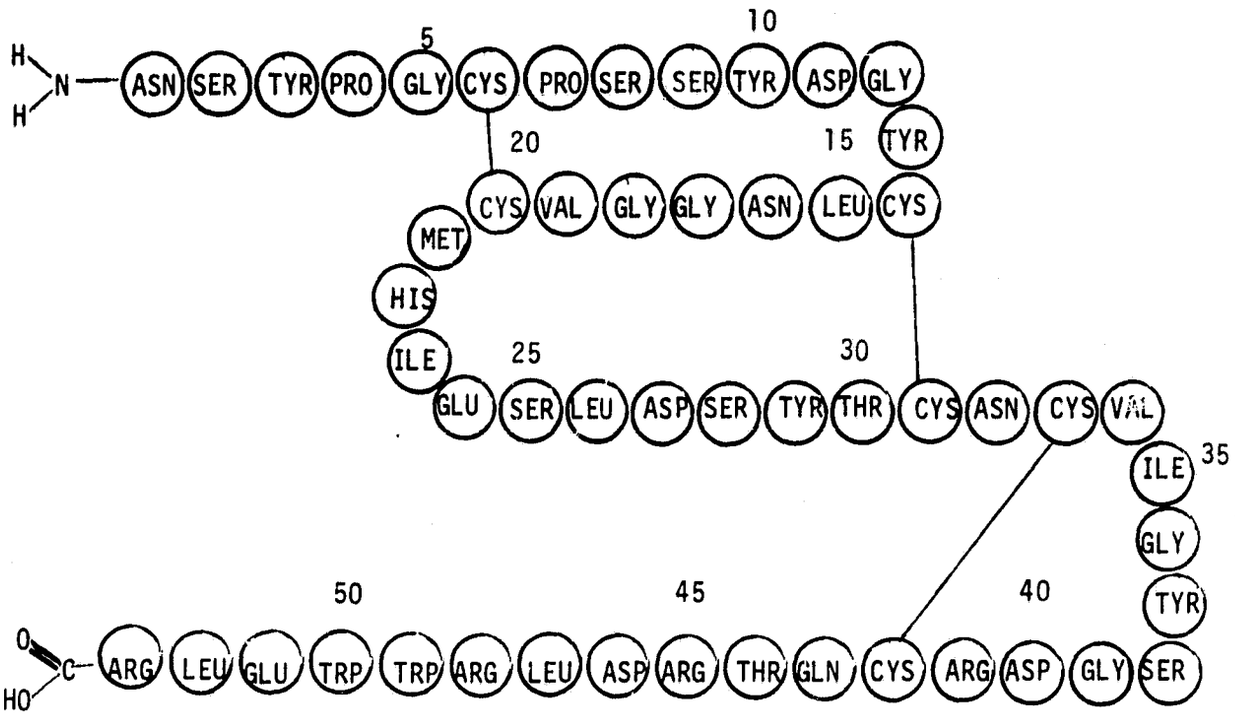

Figure 1. Amino Acid Sequence of Mouse EGF.

mobility plot developed by Weber and Osborn (7) for forty proteins. Like most growth factors, EGF can usually be found in the trailing edge of albumin or the leading edge of macro globulin peaks of plasma proteins separated by standard clinical laboratory electrophoresis techniques (16).

Using polyacrylamide gel electrophoretic technique, human EGF and mouse EGF migrate at approximately the same rate when the buffer is pH 2.3. When the buffer is raised to pH 9.5, however, the human material migrates much more rapidly (3).

d) ISOELECTRIC POINT: 4.60 for mouse EGF and 5.60 for its associated high molecular weight binding protein as determined by isoelectric focusing. The EGF isolated from human urine is anticipated to be more alkaline.

e) SOLUBILITY: It is soluble in water, urine, serum and 0.01M acetate buffer as well as dilute alkali and acid solutions. Specific concentration limits have not been reported. Cohen and Taylor conducted EGF and EGF binding protein recombination studies (2) using 0.01M sodium acetate buffer solutions containing up to 4 mg protein per milliliter.

f) SPECIFIC GRAVITY: The partial specific volume of EGF is 0.69 cm^3/gm.

g) TISSUE CULTURE: Current research has focused on the effect of EGF on other cells in tissue culture and not how to produce the factor in large quantities.

Turkington, Males and Cohen have demonstrated that EGF is present in specific tubular cells of male rodent submaxillary glands by immunofluoescent staining (8). Further staining did not detect the production of this protein in any other mouse tissue examined. These specific tubular cells are fully developed in the male only after puberty and are dependent on the sex hormonal status of the individual. Castration results in atrophy of the tubular portion of the submaxillary gland. Testosterone injections into both castrated and female mice result in hypertrophy and hyperplasia of these cells (9).

Organ cultures of murine submaxillary gland were able to incorporate labelled amino acids into a protein identified as EGF by polyacrylamide gel electrophoresis.

h) METHOD OF TISSUE CULTURE: See Section g.

i) TECHNIQUE OF ASSAY: There are currently three methods used for the assay of epidermal growth factor. The first method works in vivo and is based on the induction of precocious eyelid opening when EGF is injected subcutaneously into new born mice (6). While this bio-assy is semiquantitative at best, it can detect the presence of EGF at level of 1 microgram per gram of body weight.

A more sensitive quantitative test for EGF is radioimmuno assay using rabbit antiserum and iodinated EGF. The technique can detect the presence of EGF in concentrations of 1 nanogram per milliliter and is not affected by cross reactivity to a variety of polypeptide hormones (10, 11).

The current assay of choice is based on the ability of both mouse and human EGF to compete with I^{125} labelled mouse EGF for binding sites on human foreskin fibroblasts (14). This test has the capability of detecting as little as 2 nanograms of EGF. Each cell binds one hundred thousand molecules with an apparent dissociation constant of 2-4 x 10^{-10} M. No other peptide hormones competed for the EGF binding site on fibroblast cells obtained from mouse, rat, chicken, and human.

Stimulation of DNA and RNA synthesis in contact inhibited human fibroblasts using mouse EGF was reported for concentrations as low as 3 picomoles (15).

EXPECTED SPACE IMPROVEMENT: Because of the advanced state of purification of EGF, space can only offer the opportunity for higher throughput production of epidermal growth factor using continuous flow electrophoretic techniques. Purification may or may not be improved over current ground levels. Space may be able to offer a definite advantage to commercial availability of this product for initial research and eventual medical or biologically related use.

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NAME (GENERIC): ERYTHROPOIETIN
(PROPRIETARY): ERYTHROPOIETIN (Connaught Medical Research Labs, Toronto, Canada)

DISEASE TREATED: Anemia

PATIENTS: The kidney is known to be one source of erythropoietin production. In chronic renal failure production of this hormone is directly affected and its stimulatory effect on red blood cell production in bone marrow is curtailed. About 20,000 patients suffer from various forms of kidney failure in the United States and are on kidney dialysis machines (36). This pathology results in a severe anemia that is sometimes treated by red cell transfusions at regular bi-weekly intervals at the time of dialysis. (The average life span of a red blood cell in the human body is about 120 days and then it must be replaced.) These transfusions, however, are not used as a continuous treatment regimen because of the potential for virus hepatitis infection. Many urologists will allow their patients to remain as severely anemic as they can physiologically tolerate in order to reduce the number of transfusions received. If these patients have functional bone marrow capable of producing red blood cells, erythropoietin might be given in pharmacological doses to naturally overcome this anemia of kidney malfunction.

In addition to the anemia of renal failure, some other common anemias are likely to be amenable to erythropoietin therapy. These include the anemias associated with cancer, arthritis, lingering infections, and other chronic diseases (40). However, anemias due to blood loss or to dietary iron deficiencies will not respond to erthropoietin.

SEVERITY ESTIMATE: Erythropoietin deficiency can result in disease states ranging from chronic slight mental and physical lassitude for mild anemias to acute morbidity for renal failure patients showing transfusion reactions.

TREATMENT REGIMEN: Kidney failure - 20-200 units per kilogram body weight given once each day for the life of the kidney failure patient. (estimated dosage)

Anemia - in the less severe chronic anemias, the dosage may be the same but the rate of medication may be on an as needed basis, e.g., once or twice per year.

TREATMENT METHOD: Erythropoietin being a protein hormone must be given intravenously or subcutaneously to prevent its enzymatic destruction in the gastrointestinal tract. It is currently not commercially available as a pharmaceutical product for human use because of the current inability to purify large quantities of the protein from extraneous organic material. It has undergone a number of clinical trials in humans, however. Van Dyke (35) caused an increase in circulating reticulocytes and a 28 percent increase in circulating red cell mass twenty one days after initiation of subcutaneous injections of erythropoietin in a normal man. The treatment consisted of three injections of 147-425 units of erythropoietin given three days apart. The total erythropoietin dose was 1940 units. This patient developed redness, swelling and tenderness at the injection site but no signs of a systemic reaction. The dose estimated to produce a reticulocytosis in human subjects was 2 units per kg body weight per day for several days. The dose necessary to produce an unquestionable increase in total circulating red cell volume was estimated to be 5 units per kg per day for 7-14 days. In another study, Van Dyke and associates thought the anemia of glomerulonephritis would require a treatment level of 200 erythropoietin units per kg body weight per day (38). He administered 12,540 units of concentrated human urinary erythropoietin over 3 days to a glomerulonephritic patient with no response. (This level of product showed a positive response in normal subjects.) It is thought that uremic patients will generally tend to require higher doses of erythropoietin than normal individuals, because their bone marrow's responsiveness has been suppressed by uremic toxins. Gurney (39) using the same source material on the same type of patient gave 16,000 units over a 7 day period and observed a ten-fold increase in circulatory erythrocytes. Krantz (40) reports that Essers and associates were able to get a 2.5 fold increase in reticulocytes wnen their glomerulonephritic patients received 1870 units of erythropoietin contained in a plasma transfusion from patients with hypoplastic anemia. As in Van Dyke's patients, side effects resulting from impurities in the product stopped the clinical trails.

CURRENT GROUND STATUS: At the present time erythropoietin is not commercially available as a pharmaceutical product for human use because of the inability to both collect the raw material in production quantities and to purify that product from extraneous organic materials. No known pharmacological substitutes are available to compete with this hormone. Erythropoietin in a partially purified, lyophilized form is available for research purposes from the Connaught Laboratories in Toronto, Canada. Their current price for STEP III (see below) partially purified hormone is $0.83/unit or about $69,000 per year when applied to the treatment of a 176 pound adult with renal failure. A second commercial source of research erythropoietin is from the Erythropoietin Collection Center in the Department of Physiology of the School of Medicine, Northeast University, Corrientes, Argentina. Neither organization has filed an Investigational New Drug (IND) application for clinical trials with the U.S. Food and Drug Administration. Most researchers in this country, however, obtain the hormone in small quantities from the urine of aplastic anemic patients and attempt to purify it themselves.

It is not yet known whether there are one or more kinds of erythropoietin molecules in an animal species or even in individuals of a species; or if there are, whether these different kinds of molecules may differ in mode of action. It has not been determined whether erythropoietin acts on one or more stages of red blood cell maturation. Currently, no qualitative differences have been established in the effect of erythropoiesis between erythropoietin preparations obtained from a variety of animal species, between those from plasma and urine or between those of widely different potencies.

A number of laboratories around the world have been working to determine the source of erythropoietin in the body. A number of investigators have implicated the kidney cortex as the source of this hormone (28). Gordon (29) presented evidence indicating that under both normal and hypoxic conditions, the biogenesis of erythropoietin involves interaction between a renal erythropoietin factor (REF) also called erythrogenin and a normal serum component. The REF is found mainly in the light mitochondrial fraction of kidney homogenates which is largely composed of lysosomes. These findings suggest alternative mechanisms for the biogenesis of erythropoietin: 1) It has been postulated that the REF is a renal enzyme that activates a serum substrate possibly derived from the liver, 2) the possibility should be considered that the REF contains a pro-erythropoietin factor which is activated by normal

serum, and 3) REF contains both erythropoietin and an inhibitor of erythropoietin that is not chemically linked to erythropoietin and the inhibitor would be rendered inactive by a serum factor thus unmasking the erythropoietin activity. Antibody testing by Peschle and Condorelli (29) indicates that the erythropoietin molecule synthesized by the kidney is extracted as a pro-erythropoietin factor from the REF and unmasked from the pro-erythropoietin complex by the interaction of the REF with a normal serum component. This adjuvant finding would explain the variability of molecular weight.

a) STARTING MATERIAL AND PRODUCTION METHOD: Erythropoietin is presently obtained from two biological sources, the plasmas and urines of animals and humans. In recent years the presence of erythropoietin has been demonstrated in the normal state but the concentrations are much too low for useful preparations. Anemia can raise this concentration more than three orders of magnitude. Urine usually contains one-third to two-thirds of the erythropoietin present in an equal volume of corresponding plasma. The magnitude of the task of extracting this hormone from plasma can be appreciated when it is considered that normal plasma contains only 2.6 micrograms of erythropoietin per liter (8).

The source of human erythropoietin is the urine of aplastic anemic patients as well as those chronically anemic secondary to hookworm infestation. Patients with these conditions do not serve as donors long because of the rapid expiration of the former and the removal of the infestation in the latter. In laboratory animals, however, such as rabbits, dogs, rats, sheep, etc, the erythropoietin concentration in urine and plasma can be raised by phlebotomy, acute hypoxia, sublethal irradiation phenylhydrazine injections, or a combination of these procedures. Lowy (13) presents a series of references for each of these procedures.

A severe problem in the handling of erythropoietin is its inactivation by enzymes present in the starting materials or that arise from bacterial contamination, particularly of urines. Whenever possible, preparation of this hormone should be carried out at 2° - 5°C to minimize contaminating enzyme action and then the solutions should be stored in a freezer. For whole urines, phenol should be added to cut down on bacteria.

The production method is usually divided in steps each having their own level of purity. For the present time these steps must be carried out sequentially. The production of erythropoietin from human urine is summarized as follows (13).

STEP I. (Suitable for urine volumes ranging from a few ml to many liters) Following frozen storage, 1 ml of liquid phenol is added per liter of urine during thawing. The urine is mixed with four volumes of absolute ethanol at 2° - 5°C, allowed to stand overnight and the precipitate is collected the next day. This precipitate contains an average of 50% of the urinary activity and 80 - 90% of the urine salt.

STEP II. The precipitate is separated from the ethanol-phenol solution, mixed with water (1 gram per 2.5 ml) and the dough-like suspension transfered to dialysis bags. Dialysis is carried out at 2° - 5°C against three or four changes of distilled water over a 24 - 48 hour period. Any precipitate in the bag is removed by centrifugation and the supernatant lyophilized.

STEP III. The Step III material is dissolved in a solution of 3M sodium chloride and kept at 5°C for 50 minutes. The ratio of material to solution is 2% weight to volume. Two volumes of absolute ethanol are added at the end of that time period. One hour later the precipitate is removed by centrifugation. Activity can be found in both forms. Each is dialyzed against distilled water to remove salt and ethanol and then the dialyzands are lyophilized. The residue from the supernatant contains about half the activity of the Step II material with a fivefold increase in specific activity. The rest of the activity is in the lyophilized precipitate.

STEP IV A. One hundred mg of Step III material is dissolved in 4 ml of 0.15M sodium chloride and mixed with 4 ml of saturated ammonium sulfate. After 1-2 hours at 5°C the precipitate is centrifuged and washed with 0.9 ml of half-saturated ammonium sulfate. The combined supernatant and washing are mixed with 9 ml of saturated ammonium sulfate. The new precipitate is collected after several hours, dialyzed against distilled water and lyophilized.

Beyond Step IV A different investigators use their own procedures for further purification. These procedures have usually ranged from calcium phosphate gel extraction (17), ultrafiltration (25), Carbowax extraction (26), to gel chromatography (13). The "purified" fractions are usually checked against electrophoresis for homogeniety.

Slightly different procedures are used for the separation of this hormone from animal plasma. A detailed synopsis is given by Lowy (13). These procedures usually start with the protein extraction by boiling the plasma and then following the Steps I - III for urine, only changing the reagents or their concentrations.

Espada and Gutnisky (9) have reported that their purification of human urine erythropoietin has a potency of about 8,000 IRS units per mg of protein and was obtained in about 19% yield. Goldwasser (8) reported that he has obtained the same potency but only a yield of 0.4% from sheep plasma. Methods in use at Connaught Laboratories yield about 10,000 units per sheep in plasma (27). Their product has a shelf life of 2 years.

The difficulty in obtaining large quantities of hormone by the usual routes of urine collection and creating hypoxic sheep has started an impetus for obtaining the material by tissue culture techniques.

b) YIELD: The yield of erythropoietin from plasma and urine with present extraction methods is 0.4% and 19% respectively of that found in the corresponding biological fluid.

c) PURITY: The Connaught Laboratory material is marketed in the lyophilized form as Step I and Step III partially purified sheep plasma material. It is not marketed for human use. The erythropoietin present is only in the concentration of 2-8 units/mg, approximately 1000 times weaker than that prepared by several investigators. Heparin in a concentration of 1:10,000 may be present as a contaminant. It is unknown what other protein materials are included in this compound. Relative purity by weight of protein is calculated as 0.1% when compared to the currently most purified form available to researchers.

d) COST/DOSE (TO PATIENT) AND AVAILABILITY: Connaught Laboratories currently charges $0.83/unit for Step III purified material and $0.30/unit for Step I material. This material is sold only to investigators in bulk form with a minimal quantity requirement of 200 units. Normal delivery is 6-8 weeks after a purchase order is received.

e) STORAGE: If stored at 4°C in the powdered, freeze-dried form, the shelf life of Step I and III erythropoietin is at least three years. In solution they are stable for for four weeks if stored frozen at -20°C. Room temperature stability of solutions is a few days.

TECHNICAL DATA

a) MOLECULAR WEIGHT: A number of values for erythropoietin molecular weight appear in the literature as the result of the present inability to both obtain it in purified form and in sufficient quantities to do exhaustive biochemical analysis. The range of molecular weight is 27,000 - 100,000 Daltons. Part of this discrepancy is due to the presence of sialic acid bound to erythropoietin in plasma but not in urine. The combination of the two compounds is required for biological activity in vivo but not in vitro (tissue culture). The best molecular weight estimate of the asialo form of erythropoietin is 46,000 (8).

b) MOLECULAR STRUCTURE AND AMINO ACID SEQUENCE: Erythropoietin is a glycoprotein believed to be in the form of a single chain coiled upon itself. The shape is not known with certainty. It is tentatively described as a prolate elipsoid with an axial ratio of 3:1 when the molecule assumes a reasonable degree of hydration, i.e., 0.4 gms of water per gram (8). The carbohydrate moiety accounts for approximately 24-29 percent of the molecular structure and consists of a mixture of five different sugars. The proportion of each sugar will vary depending on the source of erythropoietin being analyzed. These differences can be seen in Table I. Sialic acid is more prevalent in the erythropoietin obtained from plasma than that obtained from urine. After sialic acid is removed, galactose becomes the terminal site on the carbohydrate moiety. Microdetermination of the amino acid composition of the protein moiety indicated that sixteen amino acids were present in urinary erythropoietin. These amino acids and their relative concentration are presented in Table I (8,9,10). The amino acid composition of the sheep plasma erythropoietin has not been determined. Whether the differences in carbohydrate composition of the plasma and urinary forms of the hormone are real must also wait for a more complete analysis to be done.

The role of sialic acid in erythropoietin appeared to be paradoxial since the asialo form had no activity when assayed by in vivo methods but had normal or increased activity when assayed by a marrow cell culture method (12). The liver apparently has binding sites for removing galactose terminal groups from the plasma circulation. If the galactose terminal group is oxidized, the asialoerythropoietin will function physiologically in vivo. The sialic acid moiety may then serve as a protective agent for erythropoietin as it circulates through the body and is removed at the site of physiological activity.

TABLE I COMPOSITION OF ERYTHROPOIETION

	SHEEP PLASMA	HUMAN URINE
CARBOHYDRATE	24%	29%
SIALIC ACID	10%	7.5%
MANNOSE	4%	(TOTAL
GALACTOSE	6%	13.0% HEXOSE)
GLUCOSE	2%	
N-ACETYL GLUCOSAMINE	4%	8.9%
PROTEIN	76%	66%
METHIONINE		1.4 moles/100 moles recovered
ARGININE		3.6
TYROSINE		3.0
VALINE		5.8
ASPARTIC		9.5
THREONINE		6.9
SERINE		6.4
GLUTAMINE		12.5
PROLINE		11.1
GLYCINE		6.2
ALANINE		7.2
ISOLEUCINE		5.4
LEUCINE		12.5
PHENYLALANINE		2.9
HISTIDINE		2.0
LYSINE		3.6

c) ELECTROPHORETIC MOBILITY: The mobility of eythropoietin in continuous flow electrophoresis has not as yet been determined to our knowledge.

Erythropoietin has been subjected to electrophoresis using polyacrylamide gel in slab and tubular forms. Step I-processed hormone showed both a fast and a slow α_1 and α_2 globulin bands when prepared from boiled rabbit plasma and unboiled sheep plasma. Albumin and β and γ globulin bands have been found in addition to these in human urinary preparations (13). Sheep plasma erythropoietin Step III has been separated into 13 bands by Allen (14). The separation was carried out at 8°C using 0.05 M tris-glycine buffer at pH=8.9 and 8 milliamps. By electrophoretic elution of the gel bands, they recovered 20 percent of the initial erythropoietin activity along with esterase activity between the albumin and α_1 globulin zone. When they repeated the same prccess with whole plasma from phenylhydrazine treated mice, the most active erythropoietin fraction represented 0.34 percent of the mouse plasma protein and 65 percent of initial erythropoietic activity. This most active fraction was resubjected to electrophoresis and divided into six bands, of which two showed hormone activity.

Goldwasser reported that his Step IV preparation of erythropoietin (having an activity of 8,000 units per mg of protein) when completely desialated, had a single symmetrical peak on gel electrophoresis pH 8.6 (15). Doing a test for heterogeneity he found two peaks at pH 6 but only erythropoietin and the asialo form of the hormone was present (8). They noted the desialated form of the erythropoietin could be fixed with sulfosalicylic acid and stained with Coomassie brilliant blue. The glycoprotein form of erythropoietin was only detectable with radioisotope labeling.

The kidney cortex cells that produce erythropoietin have also been successfully subjected to polyacrylamide gel electrophoretic separation both on the ground and in Space (16).

d) ISOLECTRIC POINT: pH = 3.3 (37)

e) SOLUBILITY: Erythropoietin is highly soluble in water (up to 18%), physiological saline, phosphate buffered saline, acids of low hydrogen ion concentration (pH=5.0-9.0) and a variety of buffers including tris and 10% polyphosphoric acid. This solubility is best when the temperature is maintained at 0°-5°C. The addition

of albumin, orosomucoid, or gelatin is reported to stabilize and enhance sheep plasma protein (17) and serum or orosomucoid might do the same thing for human urinary erythropoietin (18). The sialic acid moiety is particularily sensitive to acid hydrolysis and will split off easily. This hormone will precipitate out in the presence of high concentrations of ethanol, ether, 3M sodium chloride, saturated ammonium sulfate, 0.5M perchloric acid and 8M lithium chloride.

Erythropoietin from plasma or urine, even at very high potencies, is retained inside of Visking seamless dialysis tubing and collodion membranes during dialysis against cold distilled water or physiological saline for periods up to several days. Lewis and associates (19) reported diffusion across certain membranes at high ionic strength with dialysis lasting four days or more.

Sterilization of an erythropoietin solution can be accomplished by passing the solution through a 0.2 micron pore-size Selas Flowtronics membrane. Use of this membrane results in almost no loss of hormone activity. Sterility has also resulted by filtering the solution through Millipore membranes of the same size but the yield is drastically reduced. It is believed that the loss is the result of selective adsorption or inactivation of small amounts of erythropoietin on the cellulose esters making up the membrane. Once these esters are satisfied, the yield of the hormone returns to that found in the initial solution.

Erythropoietin can not be autoclaved at 121°C and 15 PSIG as a solid or a solution without major loss in activity. It is, however, quite heat stable and retains 25-40% of its activity after brief boiling in a neutral pH solvent.

f) SPECIFIC GRAVITY: The specific gravity of the cells producing erythropoietin has not been determined to our knowledge.

g) TISSUE CULTURE: There have been several published reports that erythropoietin can be recovered from cell culture filtrates (16, 20, 21, 22, 23). These cultures have involved bovine, rabbit and goat kidneys as well as human embryonic kidney cortex using static electrophoresis techniques in a space environment. All tissue cultures for this hormone to date have used the monolayer method for cell growth and production. These cultures have released an erythropoietically active substance into the culture media for periods up to several years but it may be the asialo

form of the hormone (23). This lack of sialic acid to make the hormone functional in vivo has led Gordon and his associates (24) to postulate the need of a kidney-liver axis to produce this hormone in vivo.

h) METHOD OF TISSUE CULTURE: A relatively pure preparation of goat glomeruli is derived from cortex by the following method (31). Using sterile precautions throughout, three stainless steel screens are placed in series, starting with #60 mesh uppermost, #100 mesh in the middle, and #200 mesh on the bottom. A fresh kidney cortex in pieces about 2 cm square is forced through the upper mesh using a spatula during rinsing with isotonic saline. The second screen will catch debris and aggregates of tubules but the glomeruli and some tubular fragments will be caught on the lower screen. Further washing of the lower screen will leave almost pure glomeruli. If this is not indicated by microscopic examination, the preparation is transferred to a tube of physiological saline and either centrifuged for 3-5 minutes at 500-1000g or allowed to sediment out. The saline is aspirated and the sedimented glomeruli are diluted with 20-25 volumes of a medium such as Parkers 199 or Eagles MEM.

An amount of the glomerular suspension just adequate to cover the growth surface of each culture vessel is added to glass or plastic flasks. The tissue cultures are incubated at 38°C in an atmosphere of 95% air, 5% CO_2 for a period of 30-60 minutes during which time numerous glomeruli attach to the surface. (Use of only saline or a medium containing serum at this stage will not allow cellular outgrowth later.) At the end of this initial incubation period the cells are drained of the first mixture and recovered with a growth medium consisting of 80% Medium 199 and 20% calf serum in addition to penicillin and streptomycin in amounts routinely used in cell culture. (Other media, such as Eagle's MEM, McCoy's 5A modified and varying types and concentration of serum including, horse, calf and fetal calf in concentrations as low as 12%, have worked in primary cultures.) Renewal of the medium every 5 or 6 days usually produces a noticable increase in proliferation. Incubation for 8-21 days results in monolayers arising from most of the attached glomeruli. Preparation from embryonic or very young kidneys usually proliferate more rapidly than those derived from adult tissue. Confluence is usually seen in 21-30 days.

Harvesting these monolayers requires scraping from the growth surface with a plastic spatula or detaching with trypsin. The glomeruli can be separated from the erythropoietin-producing monolayer by repeated pipetting with a serological pipette.

This pipetting will also break up the cell sheets for further cultivation.

Rarely is there growth before three days of incubation. Glomeruli cell cultures grow much slower than kidney cortex cells as a whole. Production of detectable erythropoietin in glomerular cell cultures appears dependent on an elapsed time interval of not less than 29-30 days between medium changes. Analysis of 19 and 25 day cultures did not show a measurable erythropoietin level. This phenomenon is seen in new and six month old cultures. Burlington and Cronkite (31) suggest that regulatory factors involved in erythropoietin production by glomerular cell cultures may have a threshold level that is only reached after a 30 day period under the culture conditions used. Ozawa (20) was able to increase his production of erythropoietin in cell culture by changing the gaseous atmosphere to 97% nitrogen and 3% oxygen.

Work by other investigators contradicts this (32, 33, 34). Tissue cultures started from mouse fetal livers produced erythropoietin between day 5 and 26 after being placed in flasks or spinner bottles (32, 33). A tissue culture of a human renal carcinoma produced erythropoietin in relatively high concentration during the first week then the output gradually declined (34).

i) ASSAY TECHNIQUES: Bioassay methods have been used to determine the levels of erythropoietin in urine, plasma, tissue extracts and other body fluids. Assay methods for this hormone have been discussed in detail (1, 2, 3, 4).

The most sensitive methods for assay of erythropoietin have been those in which the test animal has been prepared in such a way that endogenous erythropoietin production has been suppressed by either decreasing tissue oxygen demand as the result of starvation, or increasing oxygen supply with induction of polycythemia through red blood cell transfusions or hypoxic exposures. When these animals are injected with exogenous erythropoietin and a trace of radioactive iron ^{59}Fe, the iron will be incorporated into newly formed red blood cells. A measure of the amount of ^{59}Fe incorporation as compared to the same response produced by the IRS unit provides the erythropoietin concentration. The sensitivity of this bioassay method is down to 0.5-1.0 IRS units.

Because of the expense, time and amount of materials required for this in vivo assay system, a number of other in vitro assay approaches are under investigation. Immunochemical assays have included double diffusion techniques (5) and hemagglutination-inhibition of tanned red blood cells previously sensitized with erythropoietin (6). McDonnell Douglas Astronautics Company is in the process of automating the latter technique. A radio immunoassay technique for erythropoietin is also in the preliminary stages of developement (7). These immunoassay techniques appeared to be 1000 times more sensitive than other methods for detecting erythropoietin.

j) EXPECTED SPACE IMPROVEMENTS: Electrophoretic separation in space will provide a greatly increased throughput and purity of erythropoietin than can currently be achieved on earth.

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NAME (GENERIC): Granulocyte Stimulating Factor, Colony Stimulating Factor, Leukocytosis Promoting Factor, Colony Stimulating Activity

(PROPRIETARY): None

DISEASE TREATED: Agranulocytosis as found in neutropenic infections, radiotherapy and chemotherapy

PATIENTS:

The function of granulocytes is to control and remove tissue damaging material from the mammalian organism. Agranulocytosis, a condition in which these polymorphic neutrophil cells are present within the body in abnormally low concentrations, can be caused by radiation therapy, chemical toxicity and stressful situations such as massive infections and traumatic wounds. Sepsis and invasive wound infection are now the leading cause of death in patients with extensive thermal burns (16). Patients also may become severely deficient in these granulocyte cells (a condition known as neutropenia) as the result of diseases such as acute leukemia or aplastic anemia (17). Treatment of various forms of malignancies with radiation and chemotherapy will also produce this agranulocytosis. Patients subjected to surgery will also be exposed to a large quantity of material damaging to the organism. The use of CSF as a scavenger to clean up this material would greatly reduce the ravages of secondary infection and inflammation. Estimates of the annual number of potential recipients who could benefit from this granulocyte stimulating factor (CSF) vary from about 500,000 receiving radiation and chemotherapy for cancer to about two million subjected to surgery, experiencing massive burns, etc.

This CSF, of itself, is expected to be of value to patients suffering from severe infectious episodes as well as those patients in which it could be used as a prophylactic therapeutic against secondary infections. This latter situation would be of little consequence in healthy people but can result in as much as an eighty percent mortality rate in people with less than 100 neutrophil granulocytes per microliter in the blood circulation (18).

SEVERITY ESTIMATE: The diseases to which granulocyte stimulating factor may be applied with effective therapeutic value are usually those having an acute debilitating effect on the patient.

TREATMENT REGIMEN: The use of CSF is still in the experimental stage and therefore clinical dosage has not been established. For the purposes of this report we have selected a dose of 2.5×10^8 units of granulocyte stimulating factor, given several times a day until the causative condition of the agranulocytosis is removed or the patient dies.

TREATMENT METHOD: The neutrophil granulocyte occupies a central position in the body's primary defense against invasion from the outside. When bacteria break the body surfaces and gain access to the interior, granulocytes are summoned by chemotactic sense to the site of the assault. The granulocyte's role is to ingest the invader and destroy it through the action of various bactericidal chemicals liberated within the cell.

Unlike red blood cells which are almost totally within the blood stream, granulocytes are primarily tissue cells. Between five percent (17) and fifty percent (18) of the total granulocyte pool is within the circulation. Using the blood stream as a rapid means of transit to reach areas of infection or inflamation, they spend only about six to ten hours of their existence in this body compartment.

A patient's ability to deal effectively with infection would be expected to be compromised when a decrease in these granulocytes occurs. Bodey and his associates (20) suggest that the optimal therapeutic effect may require a level of circulating granulocytes near 1000 per microliter. Assuming that the patient weighs 70 kg, contains a blood volume of 4.9 liters and the life span of a granulocyte is 15 hours, then, if he is given a dose of 2.5×10^8 units of CSF approximately every six hours, he should be able to maintain a circulating level of granulocytes near the optimal therapeutic effect. The CSF would have to be given intravenously to avoid enzymatic digestion by the oral route and still provide the opportunity for rapid movement to the target cells in the marrow cavities of bone. The dosage would be continued until the problem was ameliorated or the patient died.

CURRENT GROUND STATUS: Granulocyte replacement therapy for neutropenic patients was first attempted in 1934. No clinical benefit was observed and the trials were quickly abandoned. The present success with this type of therapy suggests that these early attempts failed due to a lack of sufficient cells in the transfusion. While the current utilization of fresh granulocytes seems successful, the avail-

ability of cells is greatly limited. These granulocytes are collected from human donors by plasmapheresis using either a continuous flow centrifuge technique or filtration technique (17). Granulocytes obtained by the former method retain functional capacity in vitro and in vivo. Using the latter method, cells consistently demonstrated altered in vivo circulation but are virtually normal by in vitro criteria except for a markedly increased adhesiveness. Compatibility between donor and recipient for blood groups and granulocyte specific antigens becomes a problem both for finding donors (four different donors per day are required per patient for the duration of the treatment) and for the potential for rejecting eventual skin grafts in burn patients.

Granulocytes are usually infused through a standard blood administration set over a four hour period in order to reduce the frequency of febrile reactions in the recipient. It is not known whether the reaction is induced by antibody reacting with transfused cells or reflects the release of pyrogenic materials from granulocytes damaged during collection (17). Pulmonary problems also may be seen if the patient has potent leukoagglutinins that clump the granulocytes which then become trapped in the lung.

Another problem with granulocyte transfusions is their extremely short life span in the blood stream usually, 6 hours. This requires constant replacement of cells requiring almost continuous infusion.

To overcome the difficulties encountered with granulocyte transfusion, much work has been accomplished to determine the causative agent for granulocyte differentiation or release. CSF has been established as the causative agent for the proliferation of granulocytes from progenitor marrow cells both in vivo and in vitro. The source of CSF has not been established but it can be obtained from many different tissues of the body. While most work has been done in the area of assaying for the presence of granulocyte stimulating factor and attempting to isolate and characterize it biochemically, animal experiments have been conducted to determine in vivo biological efficacy (6). As more animal experience is obtained, eventually human clinical trials will begin.

b) STARTING MATERIAL AND PRODUCTION METHOD: The most practical method for producing CSF is to collect it from culture medium to which has been added tissues known to be high producers of granulocyte stimulating factor. At the present time human lung seems to be the most effective producer of CSF (2,3). After collecting the tissue at autopsy or from surgical excision, the tissue is immediately rinsed with Hank's or Dulbecco's balanced salt solution then minced with scissors. The pieces are then suspended in serum-free Dulbecco's modified Eagle's medium at the rate of one gram of minced lung per five milliliters of culture medium. The culture medium is spiked with 6.8 mg/ml asparagine and 3.7 mg/ml of bicarbonate. The tissue medium mixture is then divided into 200 milliter aliquots which are placed in one liter roller bottles. The bottles are incubated at 37°C in a humidified atmosphere consisting of ten percent carbon dioxide and the balance air. At the end of a three day incubation period, the medium is harvested and the cells removed by centrifugation at 9,000 g for thirty minutes. The supernatant is heat inactivated at 56°C for another thirty minutes and then the centrifugation step is repeated. The supernatant is then dialyzed against five liters of water changed every twelve hours for six times. At the completion of this dialysis step the material is filtered through a 0.45 micron filter.

One hundred milliliters of the resultant material is then passed through an hydroxyapatite column equilibrated with 0.01 M phosphate buffer at pH - 6.5. The bed volume is 50 ml. The dialyzed/conditioned medium is eluted stepwise with 0.01-0.1M phosphate buffer at pH = 6.5. Granulocyte stimulating factor will normally elute at 0.02-0.06 M concentrations of the buffer while the major protein portion of the medium will be eluted at 0.06 to 0.10 M concentrations of the buffer.

The active fractions from the chromatography step are then applied to a 6.5 percent acrylamide gel slab and electrophoresed with a 5 mM tris/glycine buffer at pH = 8.6 at 30-40 mA for fifteen hours. Eluates are collected in 2.5 ml fractions containing sufficient gelatin to give a final concentration of 0.1 percent to stabilize the CSF.

Three major peaks will be collected. The first peak is obtained in fractions 40-50 and contains about 18,200 units of CSF. The second peak is between fractions 50-80 and contains 48,230 units of CSF. The third fraction is between fractions 100-120 and contains 11,830 units of activity.

c) YIELD: From 100 ml of crude dialyzed human lung conditioned medium containing 130 mg of protein and 160,000 units of CSF activity, there is a ten percent loss of the CSF in the chromatography process and additional forty one percent loss in the electrophoretic process. Seventy eight thousand units of CSF are recovered.

d) PURITY: The material is still not completely purified, usually travelling with some contaminating protein thought to be albumin. The above process results in a 384 fold purification. As will be seen in the section on molecular weight of this substance, the material appears to be heterogeneous depending on the starting material and the method of purification.

e) COST/DOSE (to patient) AND AVAILABILITY: Granulocyte stimulating factor is not available commercially for research purposes or clinical investigation. At the present time, all CSF is produced in the laboratories of the research scientists intending to use it.

f) STORAGE: Partially purified CSF is unstable when subjected to a freeze-thaw cycle. One such cycle can result in a forty percent loss in biological activity. Stabilization during this transition period can be achieved by the addition of 0.1 percent gelatin or 0.2 mg/ml bovine serum albumin (2). Human lung conditioned medium containing CSF will show no significant loss in biological activity over a four week period when sterilized by passing the fluid through a 0.45 micron membrane filter and storing at 4°C (3).

It is stable over the pH range of 7.5-10 at 4°C for twenty four hours. Sixty percent of the biological activity will be lost when the pH of the solution is dropped to pH=3 for duplicate time and temperature conditions.

TECHNICAL DATA

a) MOLECULAR WEIGHT: A number of values for the molecular weight of granulocyte stimulating factor appear in the literature. The value is apparently dependent on the source of the material, i.e. organ and species, and the method of extraction. Using Sephadex gel chromatography to extract CSF from human lung conditioned media, Hinterberger and associates (3) found four active protein fractions having molecular weights of approximately 79,000, 40,000, 23,000, and 2,000 daltons re-

spectively. The 23,000 dalton fraction activated human marrow cells only, whereas the other fractions were active on both human and mouse bone marrow cells. These investigators imply that the 79,000 dalton fraction may be a dimer of the 40,000 dalton fraction. Fojo and associates (2) found both a 40,000 and 200,000 dalton CSF material in the same type human lung conditioned medium preparation when it was extracted with ammonium sulfate. In contrast, Metcalf found only a single fraction of approximately 25,000 daltons in mouse lung tissue (6)

Granulocyte stimulating factor found in human urine presented a molecular weight of 45,000 when using gradient gel electrophoresis or sedimentation in sucrose gradients extraction techniques. A molecular weight of 60,000 was observed on the same material using gel filtration extraction (4).

Analysis of CSF in medium conditioned by mouse fibroblast or embryo cells demonstrated a protein with a molecular weight ranging between 40,000 and 70,000 using the same extraction techniques (5).

Metcalf (6) reports that CSF produced by lung tissue from endotoxin-injected mice and extracted by sucrose gradients resulted in at least three peaks of active material with approximate moecular weights of 15,000, 30,000, and 45,000 daltons.

Two granulocyte stimulating activities have been obtained from human peripheral leukocyte conditioned medium. One fraction is in the range of 32,000-34,000 daltons and the other is less than 1,300 daltons (7).

The significance of these molecular weight variations has not been established. It may be that not the whole of the CSF molecule is required for biological activity although careful analysis of enzyme digested urinary CSF has failed to reveal breakdown products with biological activity. The existence of active sub units has not yet been disproved and clearly there can be considerable variation in chemical structure without gross changes in biological activity. A third possibility may be a series of heterogeneous proteins all working on the same target cell or cells at different stages in their life cycle. CSF is required continuously throughout colony growth. When developing colonies are transferred to cultures lacking CSF, colony growth ceases immediately and most colonies disintegrate (8).

b) MOLECULAR STRUCTURE AND AMINO ACID SEQUENCE: To date, this material has not been obtained in purified form or in sufficient quantities to do an exhaustive biochemical analysis. It is known to be a glycoprotein but its amino acid sequence has not been determined. The carbohydrate portion of the molecule contains neuraminic acid. Biological activity was still present after neuraminidase digestion (2). The protein is still functional after trypsin digestion (2) but loses its biological activity after treatment with subtilisin and chymotrypsin.

c) ELECTROPHORETIC MOBILITY: CSF from human lung conditioned medium has been separated by gel electrophoresis (2) using a 6.5% acrylamide supporting medium and 5 mM tris/glycine buffer at pH=8.6. Current was applied at 30-40 mA for 15 hours. CSF appeared well separated in the alpha globulin-post albumin region. Gradient gel electrophoretic techniques also have been used to isolate CSF (5). Progressive electrophoretic heterogeniety (with mobilities ranging from the globulin region to the post albumin region) has been observed in CSF derived from embryo conditioned medium as the incubation period progressed.

d) ISOLECTRIC POINT: The isolectric point has not been established to our knowledge.

e) SOLUBILITY: Human granulocyte stimulating factor is soluble in distilled water, tris/glycine buffer at pH-8.6, 0.01-0.1 molar phosphate buffer, a mixture of 1 mM sodium chloride and 1 mM EDTA, Hank's balanced salt solution and Dulbecco's modified Eagle's medium (2). It will precipitate in a 70% solution of ammonium sulfate.

CSF from human lung is stable at 56°C for thirty minutes in water at neutral pH but showed a sharp transition point above this temperature losing fifty percent of its activity at 60°C and all biological activity at 70°C.

f) SPECIFIC GRAVITY: The specific gravity of this protein has not been established. The density of murine myeloid progenitor cells giving rise to pure granulocyte colonies is 1.073 gms/cm^3 (1).

g) TISSUE CULTURE: The tissue origin of granulocyte stimulating factor has not been determined although the factor has been found in several human tissues, i.e. lung (3,2), spleen (9), embryonic kidney (10), placenta (11) activated lym-

phocytes (12), skin and colon (13). It has also been found in the salivary glands (14). The data so far available are consistent with two general interpretations: (a) many different cell types in the body are able to produce or release CSF and (b) CSF is produced in different tissues by a specific cell, e.g. macrophage, endothelial cell or fibroblast which is common to all tissues. The capacity of neoplastic cells or established cell lines to produce CSF may not be relevant for production.

An alternative approach has been to preincubate active cell suspensions in a liquid medium for varying periods of time and to assay this conditioned medium for colony stimulating activity.

h) METHOD OF TISSUE CULTURE: As explained above the currently most practical method for producing CSF is to obtain it from conditioned culture medium. Fojo (2) used human lung tissue obtained at autopsy to prepare the conditioned media. The tissue was rinsed with Hank's balanced salt solution and then minced with scissors. The pieces were distributed among one liter tissue culture bottles each possessing 200 ml of serum-free Delbecco's modified Eagle's medium containing 6.8 mg/ml asparagine and 0.74 gms of bicarbonate. One gram of minced lung was added for each five ml of culture medium. The bottles were incubated at 37°C in a humidified incubator gassed with ten percent carbon dioxide in air. At the end of a three day incubation period, the medium was removed and centrifuged at 9,000xg for thirty minutes. The supernatant fluid was then heat inactivated at 56°C for 30 minutes after which the centrifugation step was repeated. The supernatant was then dialyzed against six changes of 5 liters of distilled water. The dialysis changes were made every twelve hours. At the completion of the dialysis step, the supernatant was filtered through a 0.45 micron Millipore Filter.

Hinterberger and associates used a very similar technique with fresh human lung tissue (3). After mincing, they incubated the tissue in Hank's balanced salt solution for only 3-4 hours at 37°C. Five grams of lung tissue were suspended in fifty ml of solution. They found that they did not have to enrich the medium with buffers or serum. When the medium was harvested it was centrifuged at 200 g and filtered through a 0.45 micron membrane.

i) ASSAY TECHNIQUES: The most common assay for CSF is the agar colony assay using either mouse or human bone marrow as the target system. The fresh target cells are collected by sterile technique and depleted of granulocytes by passing the cells through an Isopaque-Ficoll column. The processed cells are then incubated for one hour at 37°C in a glass dish to remove adherent cells. These remaining marrow cells are plated in one ml Agar-McCoy medium, supplemented with twenty percent fetal calf serum, antibiotics and amino acids as described by Metcalf (15).

To the top of this agar plate is added 0.1 ml of the tissue conditioned medium to be assayed. If 75,000 unfractionated mouse marrow cells per plate were used as the target cells, the assay mixture is allowed to stand for seven days and then colonies are counted. Aggregates of forty or more cells are considered colonies and each colony is considered one unit. If human marrow is used as the target cells, each initial assay plate would contain 100,000 cells and the colony would be counted on day eleven because of the difference in cell generation time. During the assay development time, the plates are encubated at 37°C with 7.5 percent carbon dioxide in air and a fully humidified atmosphere.

Usually the assay is done at two levels of concentration, i.e. 0.05 and 0.1 ml/plate. When colonies are identified, they may be aspirated and stained with 0.6% orcein in 60% acetic acid for morphological identification of granulocytes.

j) EXPECTED SPACE IMPROVEMENT: Electrophoretic separation in space will provide greatly increased throughput and purity of this material than can be obtained currently on the ground. Initial separation will be required to determine the optimal tissue source and desired fraction however, before this product could be conisdered to have any commercial value.

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NAME (GENERIC): Growth Hormone, Somatotropin, Somactron, Adenohypophyseal Growth Hormone, Phyol, Phyone

(PROPRIETARY): Crescormone (Kabivitrum Company, Sweden)
Asellacrin (Serono Laboratories, Braintree, Massachusetts)

DISEASE TREATED: (1) Dwarfism as the result of pituitary deficiency
(2) Stress ulcers and the repair of gastrectomies
(3) Osteoporsis

PATIENTS:

1) There are approximately 20,000 "little" people in the United States (under 49 inches tall at maturity) according to the organization, Little People of America. Dr. J. A. Bailey (1) believes that approximately one person in 60 under 12 years of age in the United States showed the results of growth hormone deficiency in his orthopedic practice. This would amount to 840,000 potential patients. In contrast, Dr. Raiti (Director of the National Pituitary Agency) has indicated that only 1,000-1,500 patients are currently being treated for dwarfism with growth hormone in the United States (2). He feels that the total population requiring G.H. is just about being served because, 1) short statured people are aware of the availability of the hormone for theraputic purposes and, 2) the declining birth rate over the last several years has decreased the number of patients who could benefit from this compound. The number of people in the world who could benefit from such a pharmacological agent is unknown.

2) Stress ulcers are acute peptic lesions in the mucosal surface of the upper gastrointestinal tract (esophagus, stomach, and duodenum) caused by superficial loss of tissue. They usually occur when the body is subjected to a very acute physical insult such as burns, cor pulmonale, intracranial lesions, shock, and surgery. This type of ulcer responds to treatment differently than typical gastric ulcers where the stress may be psychological and of prolonged duration. Treatment of stress ulcers with growth hormone is a relatively new procedure (only done during the last six years). Experience with this treatment has been limited due to the lack of availability of growth hormone.

3) Treatment for osteoporosis is in the experimental stage. This disease presents a weakened bone structure with high potential for fractures as a result of an abnormal rate of bone resorption. It is one of the leading causes of disability in the aged, afflicting eleven million people (primarily women) in the United States (9).

SEVERITY ESTIMATE: Growth Hormone deficiency can result in disease states ranging from chronic cosmetic inadequacy for dwarfism to acute morbidity for stress ulcer patients.

TREATMENT REGIMEN: Dwarfism - two units per injection three times/week. Each injection should be spaced at least 48 hours apart
Stress Ulcers - 10 mgs/day for 4-8 days
Osteoporosis - 7 to 10 IU per day, alternated, with 100 IU calcitonin (31).

TREATMENT METHOD:

Dwarfism - Gorwth Hormone must be given to the patient before the epiphyseal plates of the bones become closed. This process usually starts at about age 10 and is completed by approximately 18-21 years of age in American males. Once treatment is started it is usually continued until the physician is satisfied that the patient has achieved average stature (10,11). This treatment usually can last about 10-12 years. Since growth hormone is a protein it must be injected either intramuscularly or directly into the blood stream to prevent its deterioration when going through the gastrointestinal tract. Because the drug is not available in an ultrapurified form it can, and often does, cause antibody formation. Approximately 30-40% of current recipients have developed antibodies to growth hormone but only 57% have stopped responding to the treatment (12).

Stress Ulcers - Winawer (3) reasoned that the defect in stress ulcers is in the mechanism controlling normal gastric cell regenerative proliferation. He treated eight patients with growth hormone using a dosage of 10 mgs/day given instramuscularly. Two patients died within 3.5 days but the other six survived and were cured when the treatment was continued for up to 18 days.

Osteoporosis - In 1974, Aloia (9) undertook clinical trials of growth hormone therapy in patients with osteoporosis, but no increase in skeletal mass was observed and the

regimen was marred by such side effects as hyperglycemia, hypertension, and the carpal tunnel syndrome. In 1977, however, Aloia's group modified their program to include not only growth hormone but dietary calcium supplements and injections of serum calcitonin, a thyroid-produced hormone which inhibits bone resorption. This combination therapy apparently overcame the difficulties encountered in the use of growth hormone alone, for an increase in skeletal mass was produced, and the side effects noted in the earlier studies were no longer a problem. In fact, Aloia comments that the restoration of positive calcium balance was of such magnitude that, if maintained, it could theoretically restore skeletal mass to normal within a few years. (31).

CURRENT GROUND STATUS: Growth Hormone is known to be produced by the acidophil cells of the anterior pituitary gland in all mammals. These cells are the most abundant in the gland and are readily distinguishable by the large number of ovoid G.H. containing granules (about 350 nm on their long axis). The hormone has been biochemically isolated, its amino acid sequence determined and producing cells cultured in small quantities for research purposes. Attempts have been made to synthesize the hormone (29,30) but Niall believes it is beyond what can reasonably be expected from current methodology. If there is an active fragment of the hormone, it must be fairly large, probably not much smaller than the whole molecule.

A major limitation to the commercial production of this hormone is the availability of human pituitaries. For G.H. to be effective in humans it must come from human tissue. Animal analogs are nonfunctional in humans (6, 19). At the present time, the National Pituitary Agency (a section of the National Institutes of Health) is the coordinator for the collection and dispersment of human pituitaries for research, diagnostic standardization and commercial production in the United States. This agency collects approximately 80,000 pituitaries per year. Serond Laboratories supplements their pituitary supply with material collected in South America and Europe.

A potentially important role for growth hormone is being evaluated in the therapy of several human disorders other than dwarfism. Our current supplies and ability to obtain the raw material to provide this product limit the availability of this hormone to the treatment of dwarfism. Either more human pituitaries have to be obtained or the pituitary glands must be cultured in large quantities and the resultant hormone production harvested to meet the demand for the product. There

are no substitutes or synthetic products anticipated over the next five-ten years to replace its pharmacological properties in the treatment of dwarfism and osteoporosis.

b) STARTING MATERIAL, PRODUCTION AND FINISHED PRODUCT: The anterior pituitaries of humans are collected at autopsy within two - three days of death. The human pituitary is very rich in G.H. and the somatotropic granules resist autolytic dissolution after death. While radioimmunoassays suggest a much higher content, the yield of somatotropin with present extraction methods is between 4 and 8 percent of the dry weight of human pituitaries equivalent to 3 to 5 mg of hormone per gland. Embalmed glands yield about half of the quantities of hormone as that from non-embalmed glands. No significant changes in the concentration of G.H. with age are evident. Octogenarians have nearly as much G.H. as the rapidly growing child (16).

Methods which have been successful for the extraction and purification of ovine and bovine growth hormones failed when applied to human pituitary glands. The techniques for fractionation of animal pituitaries is reviewed by Butt (19). The current commercial method for extraction of growth hormone from human pituitary glands was developed by Raben (20). Its success has probably been due to the fact that under the conditions employed, most of the other biologically active components are destroyed. The collected glands are homogenized in acetone and after a period of time the acetone is removed by vacuum evaporation. The resultant acetone-dried powder is extracted in a mixture of acetic acid and acetone at 70°C (to destroy bacteria and viruses). The hormone is fractionally precipitated using sodium chloride, acetone and ether. It is freed from corticotropin and melanocyte simulating hormone which are absorbed on oxycellulose. Other impurities are salted out and the hormone is finally precipitated with ethanol then isolated by electrophoretic or chromatography techniques. A time sequenced extraction procedure is outlined in Table I. Saxena has modified this procedure by extracting glycoproteins from the acetone-dried powder using ammonium acetate and ethanol and then proceding with the remaining steps.

Previously embalmed pituitary glands have to be extracted by a different method. The procedure for vialing and testing G.H. prior to shipment by the National Pituitary Agency is outlined by Raiti (6).

TABLE 1. EXTRACTION OF GROWTH HORMONE FROM PITUITARY GLAND (20)

1. Remove pituitary and store in acetone
2. Homogenize gland in acetone
3. Wash with acetone
4. Dry in vacuum

(STEPS 5 THROUGH 23 DONE AT ROOM TEMPERATURE)

5. Make acetone dried powder (100 gms) into a paste by addition of 250 ml acetone
6. Add 1600 ml glacial acetic acid and 4.8 ml water to paste
7. Heat mixture to 70°C on steam bath with continuous stirring
8. Remove immediately upon reaching temperature, stir and filter
9. Wash resultant cake on filter paper with 400 ml of glacial acetic acid and 200 ml of acetone
10. To combined filtrate add rapidly with stirring 10 ml of 5M aqueous NaCl and 1000 ml acetone
11. Store overnight at 5-6°C and remove precipitate by filtration
12. Mix filtrate with equal volume of ether
13. Allow precipitate to settle and separate using a coarse sintered glass funnel
14. Immediately wash precipitate with acetone before surface becomes dry then transfer to a dessicator and dry by vacuum pumping
15. Dissolve dried powder in 0.1N acetic acid to make 2.5% solution
16. Remove corticotropin and intermedin by stirring 8-16 hours in powdered oxycellulose (12% carboxyl content) at 20% of the weight of dried ether precipitate
17. Filter mixture through sintered glass funnel removing the oxycellulose
18. Retreat the filtrate to fresh oxycellulose (50% of weight of original ether precipitate) by stirring for 8-16 hours.
19. Remove second oxycellulose by filtration through sintered glass funnel
20. Add KOH to filtrate with vigorous shaking to make 0.3N potassium solution
21. Add glacial acetic acid immediately until permanent cloud forms at about pH of 10
22. Add 3-5N glacial acetic acid slowly to adjust pH to 8.5. (NOTE: DO NOT OVERSHOOT!!)
23. Stir mixture for 30 minutes and remove precipitate by centrifugation

(REMAINING STEPS MUST BE DONE AT 5°C)

24. Stir pH = 8.5 supernatant in ice water bath until cold
25. Add equal volume of 95% ethanol over 10-15 minutes while stirring to precipitate growth hormone
26. Continue stirring for 30-60 minutes after last addition of ethanol
27. Store mixture overnight at 5°C
28. Collect G.H. on cold sintered glass filter
29. Wash precipitate with cold 95% ethanol
30. Wash with acetone
31. Dry in a vacuum.

The finished product is a highly purified protein in the lyophilized state. The material is distributed in ampules containing 10 units each. It must be reconstituted with 5ml of sterile, distilled water and will last for one month when refrigerated at 2° - 8°C. In the lyophilized form it may be stored at room temperature. The Serono material is marketed with an impurity of 40mg of mannitol. This material is added to facilitate solubility of growth hormone in sterile distilled water and thus make subsequent intramuscular injections less painful for patients (6). The pH is adjusted between 6 and 8. Impurities in the other commercial sources are not known.

c) YIELD: The yield of somatotropin with present extraction methods is between 4 and 8 percent of the dry weight of human pituitaries equivalent to 3 to 5mg of hormone per gland (16). Embalmed glands yield about half that quantity (6).

The yield of viable cells for tissue culture purposes is 1.5 - 2.0 x 10^6 cells/pituitary (52% yield on a DNA basis). Radioimmunoassay of cell suspensions indicate 30 - 50 nanograms/1000 cells according to Hymer and Kraicer (4). This level may be increased by using G.H. releasing factors from the hypothalamus.

d) PURITY: The Serono material is marketed in the lyophilized form with an impurity of mannitol to facilitate solubility of the growth hormone in sterile distilled water. It is unknown what other protein materials are included in this or any other compound. Current purity of commercial preparations is 2 units/mg of protein.

e) COST/DOSE (to patients) AND AVAILABILITY: Serono currently charges $7.50/unit for Ascellacrin (October 1978). They do not sell the product to pharmacies or wholesale distributors. The drug is sold only to a physician who has submitted a formal request along with medical history and patient x-rays to Serono for their acceptance of the patient as a potential customer. Upon acceptance the material is shipped in four vial quantities (10 units/vial) at six-week intervals. The four vials cost $300.00. The standard shipping contract is for one year and is renewable.

The Kabivitrum Company is anticipating FDA approval to market their brand of growth hormone, crescormone, in the United States during October 1978. They are currently marketing this material in Europe for approximately $5.00/unit.

The National Pituitary Agency collects pituitary glands and contracts with several institutions to extract and purify the hormone. This material is sent to Hyland Laboratories for packaging in pharmaceutical form. The finished product is sold to companies making commercial diagnostics but distributed at $1.00/unit to research subjects through university-affiliated physicans.

f) STORAGE: Vials of commercial lyophilized growth hormone may be stored at room temperature (15 - 30°C) with minimal loss of potency. If the vials of product are reconstituted they must be refrigerated at 2 - 8°C and used within one month.

TECHNICAL DATA

a) MOLECULAR WEIGHT: 21,500

b) MOLECULAR STRUCTURE AND AMINO ACID SEQUENCE: (see accompanying chart). The hormone consists of a protein chain containing 191 amino acids with two disulfide bonds between locations 53 and 165 as well as between 182 and 189. The amino acid pattern has been identified by Niall and associates (7). The disulfide bonds are not necessary for hormonal activity or maintenance of secondary and tertiary structure (8).

c) ELECTROPHORETIC MOBILITY: Proteins in pituitary homogenates have been separated into a number of bands by starch and polyacrylamide gel electrophoresis techniques (21). Growth hormone was one of the first hormones to be separated by this technique. G.H. is ordinarily associated with the furthest cathode band. Hodges and McShane have also conducted successful electrophoretic separations of the granules containing growth hormone in pituitary acidophil cells (5).

d) ISOELECTRIC POINT: 4.9

e) SOLUBILITY: Sparingly soluble in water (0.03% at pH 7.1). It is soluble in aqueous solutions of urea, ammonia and alkalies. Hormonal activity is lost completely in boiling water within 10 minutes (19). A 1% G.H. solution in aqueous 6.66M urea (at pH 7.0) did not lose any biological potency.

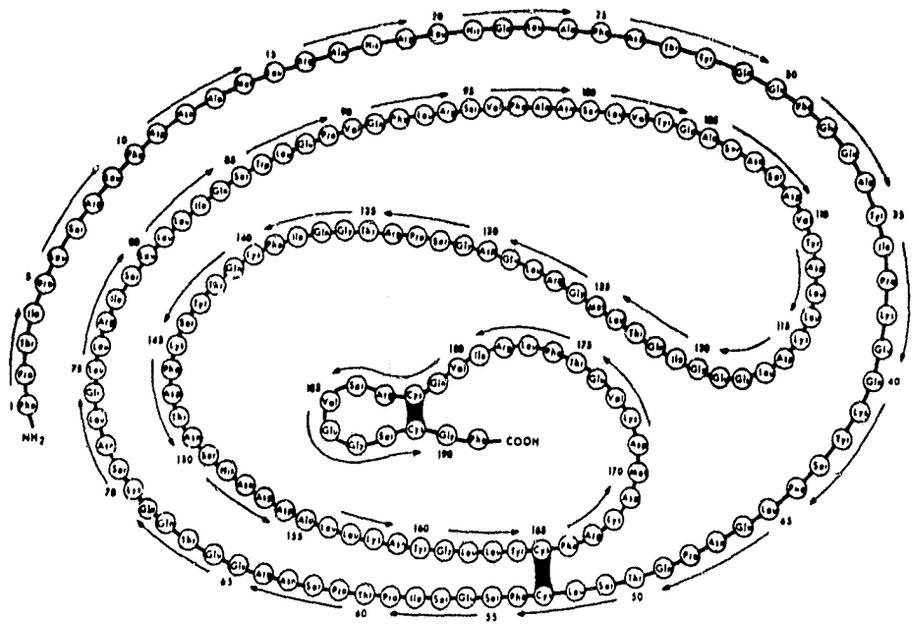

Figure 1. The Primary Structure of hGH.

f) SPECIFIC GRAVITY: 1.0705 - 1.0850 with 80-92 percent G.H. producing cells. The variation is interpreted to be the result of the quantity of granules in each cell.

g) TISSUE CULTURE: Kohler (22) showed that tissue cultures of pituitary cells did incorporate radioisotopes into new growth hormone. This indicated complete automony of the cells for producing this hormone. Several clones have produced G.H. for several years after isolation of the cells to form clones (25). Generation time is between 20 - 30 hours. These cultured cells rapidly become heteroploid according to Sonnenshein but still produce hormone (23). Bancroft (24) reports the hormonal content of each cell is low but is rapidly renewed, i.e. about every 15 minutes.

Tissue culture of pituitary tumors producing G.H. do so at a higher level and for a longer time than culture of normal human pituitary cells according to Tixier-Vidal (25). Hypothalamic extracts will stimulate G.H. production by the pituitary.

An interesting observation that is undergoing confirmatory studies was reported by Yasumura. His tissue cultures of rat growth hormone producing cells, in operation for several years, are also producing a second hormone, prolactin. He postulates the same cell is producing both hormones (26).

Batzdorf grew suspension cultures of trypsinized human cells for five weeks. G.H. production was at a maximum between the 7th and 14th day and decreased to undetectable levels after five weeks (27). In general, tissue culture cells are smaller (about 10 microns) and contain less secretory granules. Hormone production rate is high initially but drops off after 1-2 months to stabilize at a lower level.

Attempts have been made to estimate G.H. secretory rates on the basis of basal G.H. levels and their metabolic clearance. This calculation ignores the marked fluctuation of plasma hormone which does occur throughout the day and night as well as with exercise and stress. Integrated over a 24 hour basis, Daughaday suggested that normal G.H. secretion is between 0.75 and 3mg/day (16). Radioimmunoassay of cell suspensions indicated 30 - 50 nanograms/1000 cells according to Hymer and Kraicer (4). This level may be increased using growth hormone releasing factors from the hypothalamus when grown in suspension culture.

h) METHOD OF TISSUE CULTURE: A cell dissociation procedure, applicable to the pituitary gland has been developed by Hopkins and Farquhar (28). The procedure is outlined in Table 2. This procedure takes only 45 minutes, yields greater than 90% single cells and produces 1.5 - 2.0 x 10^6 cells/pituitary (55% yield on a DNA basis). Ninety-five percent of the cells are viable based on trypan blue exclusion. It should be noted that this procedure is expected to partially degrade the cell surface components and that a period of 3 - 5 days is necessary for their regeneration and repair. The additional proteins synthesized by acutely dissociated cells and isolated by gel electrophoresis may be related to these repair processes.

Tixier-Vidal (25) has reviewed the current work in cell culturing of growth hormone. Sixteen references are provided for greater detail in the methods of monolayer and suspension culture of these cells.

i) TECHNIQUE OF ASSAY: The biological activity of growth hormone preparations is occasionally assayed by the ability of that hormone to stimulate body weight gain in hypophysectomized rats (18) or in female rats whose growth has reached a plateau (17). These tests are very slow usually taking 5 days to 3 weeks to get a result. A more sensitive method for assaying G.H. depends upon the administration of the hormone for 3-4 days and then measuring a change in the width of the tibial epiphyseal growth plate. The minimum dose required to achieve a significant response is inadequate for the quantitative measurement of this product in biological fluids.

TABLE 2. PITUITARY CELL DISSOCIATION PROCEDURE (28)

1. Mince pituitary gland into about 40-50 small pieces
2. Incubate pieces in 0.1 mg/ml trypsin for 15 minutes, then immediately centrifuge and drain
3. Incubate in 5 µg/ml DNAase for 5 minutes, then immediately centrifuge and drain
4. Incubate in 2 mg/ml Soya bean trypsin inhibitor for 5 minutes, then immediately centrifuge and drain
5. Rinse cells in calcium free Krebs-Ringer-Bicarbonate solution
6. Incubate in 8 µg/ml neuraminidase (Clostridium perfringens) and 1 mM EDTA in calcium free Krebs-Ringer-Bicarbonate for 15 minutes
7. Rinse twice in calcium free Krebs-Ringer-Bicarbonate solution

   (NOTE: All incubating media contain 0.3 percent BSA, 14 mM glucose and a complete amino acid supplement in the calcium free Krebs-Ringer-Bicarbonate solution.)
8. The cells can be completely dispersed by mild pipetting in the incubating media. The fact that the pieces hold together during incubation and washing greatly facilitates their handling.

For clinical purposes, G.H. is measured by one of several radioimmunological methods (13, 14, 15). Results are expressed in terms of a highly purified G.H. preparation. The immunological activity of this product does not correlate completely with biological activity (16).

EXPECTED SPACE IMPROVEMENT: Because of the advanced state of purification of this hormone, space can only offer the opportunity for higher throughput production of growth hormone using electrophoretic techniques. Purification may or may not be improved over current ground levels. Space may be able to offer a definite advantage to commercial availability of this product as the result of anticipated improvements in cell culturing in the weightless state.

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NAME (GENERIC): Interferon

DISEASE TREATED:
1) Chronic Hepatitis
2) Sarcomas and Carcinomas
   Hepatoma
   Breast Cancer
   Osteo Sarcoma
   Hodgkins Disease
   Multiple Myeloma
   Juvenile Laryngeal Papilloma
   Lymphoblastic Leukemia
   Transitional Cell Carcinoma
3) Viral Infections
   Influenza
   Herpes Zoster
   Common Cold

PATIENTS: Interferon appears to be the modern day panacea for almost all diseases of known viral origin as well as several other diseases in which viral involvement has been suspected but not proven. The clinical use of interferon is still in the experimental stage with only a small part of its therapeutic potential explored.

1) Chronic hepatitis is a disease of viral origin that can affect approximately 100 million people world wide (2). This virus is usually transmitted by means of sewage contaminated water supplies and unsanitary living conditions. In the United States alone, about ten percent of the patients hospitalized with serum hepatitis, hepatitis B, become chronically infected with a different virus. People that receive blood transfusions and passive immunization injections may be inflicted with the disease. Clinical trials with interferon have shown that it appears to interfere with the multiplication of the causative virus particles and thus suppress the disease.

2) In the United States alone, 383,000 people died in 1977 from all forms of cancer (26). Many more are subjected to radical surgery, radiation and chemotherapy in an effort to remove and/or stop the spread of cancer after it has been detected. The "single commonest cancer on earth" is primary hepatoma, according to Dr. W. A. Carter, Chairman of Medical Viral Oncology at the Roswell Park Memorial Institute in Buffalo, New York (23). Reporting that there are over 200 million victims of this disease, he indicates the linkage between hepatoma and virus hepatitis B is statistically stronger than the link between lung cancer and smoking. The use of interferon as an antitumor and anti-viral agent has shown great promise in clinical trials over the last several years.

3) While the common cold, herpes zoster, and influenza have all responded to interferon treatment, the most effective use of this product may be in the prophylactic treatment of people whose immune system is absent or has been depressed by drugs. This situation, among others, arises in the steroid treatment of arthritis chemotherapy given to prevent rejection of tissue and organ transplants and in chemotherapy of certain types of cancer that do not respond to radiation treatment.

SEVERITY ESTIMATE: The diseases for which interferon has known therapeutic value range from the acute malaise of influenza and common cold through the chronic debilitating conditions of hepatitis to the terminal conditions of osteo sarcoma and leukemia.

TREATMENT REGIMEN: Chronic Hepatitis - 100,000 units/kg intravenously daily for the first two weeks and then cut the daily dose in half every two weeks until 300 units/kg is reached (2).

Breast Cancer - 3 million units/day directly into the lesion for 1-2 months after metastatic modules are observed. Another study has shown success with 1 million units/day intralesionally for 10 days (23).

Osteo Sarcoma - 3 million units/day intramuscularly for 30 days after amputation or wide resection of the tumor bearing limb followed by 17 months at 3 million units three times per week (23).

Juvenile Laryngeal Papilloma - 3 million units intramuscularly given 3 times per week (23).
Lymphoblastic Leukemia - 1 to 2 million units/kg/day (23).
Common Cold - 14 million units divided into 40 doses given over a 4 day period by nasal spray.

TREATMENT METHOD: All of the above treatment regimens are those reported by individual clinical investigators while doing therapeutic research on a particular disease. This will account for the large diversity in dosage for treating metastatic breast cancer. The actual dosage for any of the diseases above has not been standardized and must await the collection and analysis of data from further human clinical trials. Interferon is not presently commercially available as a pharmaceutical product because of the current inability to produce and purify large quantities of the material.

For chronic infections with hepatitis B, Merigan and his associates at Stanford University initially treated their patients with very heavy doses of 17 million units/kg of body weight daily for a week followed by a second series of interferon injections at half the concentration two weeks later. While these short courses of interferon therapy had an obvious but transient effect on reducing DNA polymerase in the patients' blood, a sustained daily treatment consisting of constantly reducing doses at biweekly intervals resulted in a prolonged suppression of the hepatitis B activity at the same time reducing the presence of associated antigens in the blood.

Dr. Gutterman at Anderson Hospital and Tumor Clinic gave interferon therapy to nine women with metastatic breast cancer after they did not respond to conventional chemotherapy. Daily injections of three million units of interferon directly into the lesions for a period of 1-2 months produced a measurable regression of the lesion in five of the women. While there were no local reactions at the injection sites, some of the patients reported minimal side effects consisting of fluctuations in body temperature, loss of appetite and slight fatigue. Bone marrow suppression was observed in those who had already been given chemotherapy necessitating temporary reduction of the interferon dosage.

Dr. Habif at Columbia University in New York observed a marked lesion regression in six women using only one million units/day over a ten day period for the same disease. Using foreskin fibroblast interferon rather than leukocyte interferon as reported above, Dr. Carter at the Roswell Park Memorial Institute treated a post-mastectomy patient with metastatic lesions. He injected 500 thousand units daily into one of four nodules and a placebo in three others. The treated nodule regressed even though it had previously resisted hormonal and chemotherapy.

About 80% of the patients with osteo sarcoma develop lung metastases within a year after surgical removal of the initial tumor. The survival rate two years after contracting the disease is about 35%. Dr. Strander at the Karolinska Hospital has been treating more than 30 patients with this disease over the last seven years. After amputation or wide resection to remove the primary tumor the patient is immediately started on 3 million units of interferon intramuscularly per day for one month. At that time the treatment is reduced to three times per week at the same dosage on an outpatient basis. Only 36% of his patients have developed the lung metastases at two and a half years after the operation with a 73% survival rate.

Treating three small children with relapsed acute lymphoblastic leukemia, Dr. Hill of the Wadley Institute of Molecular Medicine has been giving 1 to 2 million units of interferon per kilogram body weight per day. This treatment substantially cleared the peripheral blood of lymphobasts within twelve days and the bone marrow of one child within four weeks.

CURRENT GROUND STATUS: In 1957 Isaacs and Lindenmann discovered a biochemical agent that was released by cells into their surrounding medium when infected by a virus. When this medium, free of cells, was added to another cell culture, the new cells became resistant to viral infection. This agent, designated interferon, did not react selectively with a particular free virus particle similar to an antibody reacting with its particular antigen, but only with cells which then became resistant to a large number of different viruses. Interferon however was species specific; interferon induced in rabbit cells would not offer viral protection to human cells. Because it was a natural cell product, pharmacologically

active against many viruses yet unlikely to harm cells or provoke resistance in exposed viruses, interferon's ability to be harvested from tissue culture gave the potential of great clinical significance.

This potential diminished for a while because the standard biochemical isolation methods did not yield a homogeneous product that could be described as a single protein. Paucker (18) achieved a degree of purification using antibody to interferon in an affinity chromatography column. Others have also obtained some purification using albumin and lectins as binding materials on the chromatography column. Stewart (14) found that boiling the interferon in sodium dodecyl sulfate (SDS) made it possible to separate interferon from other proteins to which it was adsorbed. Removal of the SDS allowed the interferon to recover its biological activity. If a reducing agent were added to the SDS-interferon mixture and then both the SDS and reducing agent were removed, two types of interferon were present. The major component, designated "Le" or leukocyte interferon (because of its source) was not renatured. The "F" type of interferon was biologically active with or without the reducing substance being present during detergent treatment. This "F" type leukocyte interferon is very similar to the interferon induced in foreskin fibroblast cells. Several investigators have recently claimed the achievement of homogeneity for their isolated material but they have not accumulated enough material for characterization.

After it was discovered that many viruses, normal and attenuated, could induce interferon in almost all cells both in vitro and in vivo, it was also found that other agents could achieve the same purpose. Any substance that was equivalent to a double stranded RNA was capable of interferon induction, e.g. two antibiotics (helenine and statolon), a synthetic RNA/polyinosinic acid-ribocytidylic acid) as major inducers. If, after induction, the cellular RNA is removed and inserted into cells of a different animal species which is then challenged with a virus, the second culture of cells will make interferon specific for the species of animals providing the original RNA. Interferon has also been made in test tube quantities at the National Institute of Health in a cell free protein synthesizing system containing a mixture of cell extracts. Carter (24) believes that neither organic synthesis nor genetic engineering will produce practical quantities of interferon for at least a decade.

After the initial clinical trials it became obvious that large scale production and purification of interferon was going to be a major problem. Some pharmaceutical companies explored products, like poly I:C, to serve as inducers of interferon in vivo. Although this stimulated the production of large quantities of circulating interferon in rodents, the concentrations were less in primates and humans. The inducers also caused serious side effects resulting in the abandonment of this pharmacological approach. Researchers at Roswell Park Memorial Institute and Johns Hopkins University have teamed up to develop a nucleic acid that triggers interferon induction endogenously but will be destroyed within several minutes after injection thus eliminating side effects. It is hoped that this material will go into clinical trials in 1979.

One of the clinical drawbacks of the fibroblast interferon is its systemic instability. It must be administered intravenously because it does not diffuse readily into the blood stream from an intramuscular injection site. A number of laboratories are working this problem trying to alter the pharmacokinetic properties of this interferon.

The American Cancer Society has recently announced the start of large scale clinical trials to test interferon activity against various types of cancer. The FDA has approved both leukocyte and fibroblast interferon for IND clinical trials.

A number of chemotherapeutic products are already on the market for treating cancer and certain types of virus. While they offer some pharmacological protection to recipients many patients demonstrate physiological resistance to these compounds. Thymidine is the latest anticancer competitor to enter clinical trials. If interferon can be made in sufficient quantities to make it commercially attractive it may become the drug of choice for any disease for which it provides therapeutic value.

The W. Alton Jones Cell Science Center is about to publish the papers of a 1977 symposium on human interferon production and its use (24). This symposium dealt with methods of mass production of human interferon as well as its standardization, safety, and purification.

b) STARTING MATERIAL, PRODUCTION AND FINISHED PRODUCT: Although almost any cell can produce interferon, current clinical trials have relied on only three tissues: 1) the human leukocytes collected from blood donors, 2) fibroblasts grown in tissue culture from human infant foreskin designated the FS-4 strain, and 3) the human leukocytes from a high yield lymphoblastoid Burkitt's lymphoma cell strain.

The standard method for producing interferon from white blood cells is to:

1) pool whole blood from a number of human donors
2) centrifuge to separate the blood into its different cellular constituents and plasma
3) aspirate off the buffy coat of white blood cells
4) wash leukocytes with physiological saline several times to remove any contaminating erythrocytes
5) suspend leukocytes in a tissue culture medium containing 5% fetal calf serum and 2.2 m M glucose
6) upon achieving culture size desired, innoculate the culture with attenuated Newcastle Disease Virus or Sendai Virus at the rate of about 10 pfu/cell.
7) Incubate the mixture at 37 deg C for up to 24 hours.
8) Return the mixture to room temperature and remove the virus by draining the culture media.
9) Wash the cell cultures three times with culture media.
10) Add culture media, adjust to pH = 2 with 5N Hcl and let stand several hours at 2 deg C to inactivate any residual virus.
11) Drain the culture media at 8-10 hours after induction.
12) The drained culture media should be neutralized with 5N NH_4OH.
13) Purify according to a variety of techniques.

Interferon can be produced both by stationary monolayer cultures and suspended cell cultures. The Cell Culture Center at M.I.T. uses dextran beads 160 microns in diameter for attachment of foreskin fibroblasts in a suspended cell culture interferon production system. The use of these beads increases the surface area for cell attachment about six times, thereby enhancing interferon production by the culture.

A number of factors influence the rate of production of interferon. This area is given very thorough treatment in the chapter on formation in cell cultures in the book by Solovev and Bektemirov (4). It should be noted that the quantity of interferon produced by the cells depends on the age of the culture, type and dose of inducer virus, length of exposure to the inducer, incubation temperature, number of cells, hydrogen ion concentration and the time after induction that the interferon is harvested. The maximum production of interferon will take place between 8-10 hours after induction. The product will not appear in the culture medium until 4-6 hours after induction. After about 10-12 hours the presence of interferon in the medium acts on an apparent feedback mechanism to reduce production. Priming the producing culture within 10 hours of induction will also lower total production. A second induction period with either a virus or an anti-metabolite six hours post induction will cause a slight increase in interferon production.

After the cells are induced to produce interferon many of the cells will die (usually about 70%). In the case of Ehrlich ascites tumor cells induced with Newcastle Disease virus (5) the surviving cells produced neither significant amounts of interferon nor cytotoxicity. Resumption of growth began about the third or fourth day after induction. When the cells were reinduced to produce interferon a second time about 56% of the cells died and the only about 21% of the initial interferon production rate returned. Further work by Patrovic and associates (5) indicated that the level of inducer required to produce interferon may be lower than that which causes toxicity.

The length of cell culture exposure can be shortened considerably to minimize cytotoxicity Patrovic and associates use an exposure time of ninety minutes (5). Carter (23) advocates a three minute induction period.

There are several methods for purification of the harvested interferon, none of which currently yields a homogeneous protein. Starting with a harvested crude interferon containing about 20,000 units/mg protein Paucker (18) sequentially processed the material through: 1) ultrafiltration, 2) dialyses against 0.01M sodium acetate at pH = 4.5, 3) adsorption onto carboxymethyl-Sephadex C-25 at pH = 6 in 0.1M phosphate and elution in a rising pH gradient between pH = 6.8

and 7.7. This resulted in a fifty fold increase in purification. This material was then subjected to polyacrylamide gel electrophoresis for a purity improvement by at least a factor of five. To achieve this level of purity, he started with a harvested culture medium that was high in interferon yet relatively free of contaminating proteins. This was accomplished by both bathing the rotating monolayer cultures with a minimal amount of fluid that contained no serum and only collecting the medium during the interval of its maximal release from the cells.

c) YIELD: The recovery of partially purified interferon obtained by the purification procedure followed by Paucker (18) is 10-20 percent before the final polyacrylamide electrophoresis step.

Levine (25) is cited as producing 1 international unit of interferon per 1000 cells in both roller flasks and dextran bead support suspension cultures. Personal contact with the M.I.T. Cell Culture Center indicated that the values in the article are incorrect. Based on their data, fifty cells should produce one unit of interferon. This is accomplished using a superinduction system incorporating poly rI:rC, cycloheximide and actinomyocin D.

d) PURITY: The material used in human clinical trials has come almost exclusively from Helsinki and is of the leukocyte type interferon. Currently, in its most refined form, the active interferon has a concentration of one million units/mg of protein and constitutes about one part per thousand of the soluble material actually supplied for clinical purposes. The contaminating materials are protein in nature and have not been identified because they will vary with the source of interferon production. The leukocytic type interferon is produced from pooled donor blood. It probably contains some viral fragments from the challenge virus and other products due to any chromosomal variation within the culture of pooled white cells. Foreskin fibroblast interferon, on the other hand, is derived from a purified strain tissue culture resulting in a relatively constant set of contaminants. The difference in contaminants between the two types of interferon can be seen clinically; the chance for a toxic reaction against myeloid tissue is more frequently observed if interferon originates from a leukocyte culture rather than a foreskin fibroblast culture. Interferon molecules also have a tendency to attach to other molecules and thus be contaminated with them when separated.

Lin and his co-workers (1) at the International Laboratories for Molecular Biology of Interferon Systems have reported their purification of human leukocyte interferon to apparent homogeneity but in quantities as yet too small to identify biochemical constituents and sequence. Knight reports that he has obtained human fibroblast interferon in probably homogeneous form.

Burke (2) believes the specific activity of interferon is apparently one billion units/mg.

e) COST/DOSE (TO PATIENTS) AND AVAILABILITY: The current cost for leukocyte interferon is $50/million units from the Interferon Department, Finnish Red Cross Blood Transfusion Service in Helsinki. It is the largest supplier of interferon at the current time with an annual production of about 100 billion units. About one fourth of this production over an 18 month period has been ordered by the American Cancer Society for a large scale clinical trial. Sloan-Kettering Institute through a private foundation grant has started production of human leukocyte interferon in Switzerland and hopes to match the Helsinki annual production in 1979.

The Wellcome Research Laboratories in England has recently announced the large scale production of interferon induced from lymphoblasts cultured in 1000 liter fermentation vats. Annual production is also expected to pass that accomplished by Helsinki in 1979.

HEM Research, Rockville, Maryland is the only commercial source of any type of interferon in this country. Like the Massachusetts Institute of Technology Cell Culture Center and the Roswell Park Memorial Institute, it is producing interferon from foreskin fibroblast tissue cultures. Calbiochem will be marketing interferon from various tissues for research purposes only.

Because of the tremendous medical market potential for this compound, approximately 100 industrial and university laboratories around the world are making interferon for their own research purposes. The companies include Imperial Chemical Industries and Glaxo Laboratories in Great Britain, Sandoz in Austria as well as E. J. Dupont de Nemours, G. D. Searle and Merck, Sharp and Dome in

America. With continued clinical success, the interest and technical skills of these laboratories will ultimately increase production and lower unit cost.

While interferon from all of the above sources is only available for research and some FDA authorized human clinical trials, none of the material is available to the average private physician for treating pathology. In Russia, however, Friedmann (3) indicates interferon is an over-the-counter product. He does not indicate its cost but does suggest that it is not obtainable in sufficient quantity to be of significant clinical value to a patient.

In the United States, all interferon for clinical research purposes must be obtained through the Interferon Working Group at the National Cancer Institute.

f) STORAGE: A considerable amount of research in this area has been reported by Solovev and Bektemirov (4). About 85% of the human leukocyte interferon activity is lost after being heated to 56 deg C for one hour. When the same material is kept at 4 deg C for up to eight months it will not lose its activity. Freezing to -40 deg C and then thawing at 4-10 deg C through three cycles did not decrease the pharmacological activity of the material. Lyophilyzation and subsequent storage of interferon at 4 deg C for 18 months was not accompanied by any decrease in biological activity.

Human interferon is stable between pH = 1.0 to 10.0. This resistance of interferon to strong acids (for at least 7 days) is used to inactivate inducing virus in the interferon production process (5).

Purified preparations of interferon have marked sensitivity to ultraviolet radiation (6) but exposure to x-radiation for 10 minutes does not reduce biological activity (7). It is resistant to ultrasound (4).

TECHNICAL DATA

a) MOLECULAR WEIGHT: At the present time there are three types of human interferon used in clinical trials, each with its own molecular weight and possibly different chemical composition (15). "F" type interferon originates from foreskin fibroblasts and has a molecular weight estimated to be about 20,000. The white blood cells produce two different types of interferon. "Le" interferon is

the most abundant type and has a molecular weight of approximately 15,000. The other component, leukocyte interferon type II, has a molecular weight of approximately 21,000 and is produced by the "T" lymphocytes of the immune system.

Interferon induced in human amnion cells has been reported to have a molecular weight of 160,000 (8). To our knowledge it has never been used in human clinical trials.

Reports in the literature indicate that the molecular weight of interferon may depend on the type of tissues in which it is induced, the material used to induce it, the location of production in cell culture or in vivo in a particular animal species (4). The molecular weight range reported for any combination of the above conditions varied from 15,000 to 160,000.

It must be noted that interferon has a well marked species specificity. Earlier reports in the literature that this specificity was not absolute (9) are attributed to the presence of other inhibitors or the interferon inducing activity of residual virus in the early interferon preparation (9, 10).

b) MOLECULAR STRUCTURE AND AMINO ACID SEQUENCE: The various interferons have not been isolated in sufficient purity and quantity to determine their molecular structure and amino acid sequence. Current literature reports interferon to be a glycoprotein consisting of one or more side chains ending in sialic acid (11). The backbone of the molecule is a folded polypeptide of one or more chains linked by disulfide bonds (4). It is known to contain tyrosine, tryptophan, arginine and lysine with neither of these amino acids exceeding eleven percent (6). It is not known to contain nucleic acids.

It is assumed that each type of human interferon has a common sequence of amino acids for at least some portion of the molecule because of the slight ability to protect against viral attack in other species with human leukocyte interferon.

The use of sodium dodecyl sulfate and reducing agents to temporarily denature interferon has suggested the protein has at least a secondary structure (12, 13, 14, 16).

c) ELECTROPHORETIC MOBILITY: The various interferons do carry an electrical charge (17). Polyacrylamide gel in one and two dimensions (18, 19), isoelectric focusing and isotachophoresis (18) have all been used as methods to purify interferon with varying levels of success. Paucker (18) reports that the duration of the isoelectric focussing procedure causes some of the interferon to be inactivated. In most cases the quantities processed have been so small that it is very difficult to get meaningful data without using additional markers such as radioisotopes, antibodies etc.

d) ISOELECTRIC POINT: Two ranges have appeared in the literature. Lampson and associates (20) report a range of 6.9-7.1 while Bostandzhyan and Balezina (21) indicate 7.2-7.8. This variation may be explained by the source of the interferon production. Interferon induced by Newcastle Disease Virus in tissue cultures of mice spleens showed an isoelectric point of 7.0 as compared to an isoelectric point of 7.4 and 7.7 for the two interferons found in mouse sera after challenge with the same virus (4).

e) SOLUBILITY: Interferon is soluble in water, 6M urea, 5N ammonium hydroxide and 0.1M phosphate. It can withstand $NaIO_3$ at room temperature for one hour in final concentrations ranging from 0.001 to 0.00001 M. Ammonium sulfate will precipitate interferon but it retains its biological activity after redissolving in water. Interferon can also be precipitated with ethanol, acetone, zinc acetate.

Interferon does not dialyze (4, 18).

f) SPECIFIC GRAVITY: Paucker (18) reports the buoyant density of L cell mouse interferon with a molecular weight of 23,000 as 1.3 gms/cm^3 in cesium chloride.

g) TISSUE CULTURE: Interferon can be induced in almost any cell of the human body. Any large scale tissue culturing technique would be acceptable for innoculation with a challenge virus to produce the interferon. A brief description of the method for culturing both cells and challenge virus as well as inducing interferon is described by Patrovic and associates (5).

h) METHOD OF TISSUE CULTURE: See Section g.

i) TECHNIQUE OF ASSAY: The standard method for bioassay of interferon is to measure its ability to protect cells in tissue culture against a challenge virus. The common interferon assay unit is that amount that will protect 50% of the cells in a tissue culture from destruction by vesicular stomatitis virus. Cell viability is determined by the standard trypan blue exclusion method and is expressed as the percent of the total number of viable cells found on a control that had not been exposed to the virus or an inducer.

In a highly purified interferon preparation having a specific activity of 200 million units/mg, one biological unit would probably weigh 0.005 nanograms/ml.

The National Institute of Health has established a mouse reference unit (No. G-002-904-511) for comparison purposes among laboratories.

EXPECTED SPACE IMPROVEMENTS: Space may definitely offer improvement in purification and throughput for interferon production. Anticipated improvements in cell culturing in the weightless state may increase cellular production of the material. If different pathogenic virus strains remain necessary to induce production, an interferon production facility in space would minimize safety problems on the ground in case the virus becomes uncontrolled.

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NAME (GENERIC): IMMUNE HUMAN SERUM GLOBULINS

(PROPRIETARY): AR-TET (Armour Laboratories, Kankakee, Illinois)
Gamma Globulin (American Red Cross)
Gammastan (Cutter Laboratories, Berkeley, California)
Gammar (Armour Laboratories, Kankakee, Illinois)
Gamulin (Dow Pharmaceuticals, Indianapolis, Indiana)
Hyparoitin (Cutter Laboratories, Berkeley, California)
Hypertussis (Cutter Laboratories, Berekely, California)
IMMU-G (Parke-Davis, Detroit, Michigan)
Immuglobin (Savage, Houston, Texas)
Immune Serum Globulin (Lederle, Pearl River, New York)
Immune Serum Globulin (Abbott Scientific, Chicago, Illinois)
Tetanus Immune Globulin (Abbott Scientific, Chicago, Illinois)
Tetanus Immune Globulin (Wyeth, Philadelphia, Pennsylvania)

DISEASE TREATED: For the purposes of this report we have grouped a number of these protein antibodies together into a common class of immunoglobulin products. This group includes, but is not limited to, protein antibodies against rubella, rubeola, mumps, poliomyelitis, varicella, diphtheria, pertussis, tetanus and hepatitis.

PATIENTS: About 309,000 cases representing a tabulation of the above mentioned communicable diseases are reported to the National Center for Health Statistics (1) annually in the United States (see Table I). While a dose of specific immune serum globulin may be given to modify or minimize the effect of that specific disease in a person already afflicted, the usual procedure is to immunize all the people who have or may come into contact with the patient until the incubation period has been exceeded. Depending on the seriousness of the disease, the number of subjects receiving this passive immunity can range from 1-6 close contact family members for mumps up to many thousands of individuals threatened by serious epidemics. The number of people given passive immunity from year to year will depend on many variables, but primarily is related to the number and location of people afflicted. When the city of Austin had an outbreak of diphtheria in 1967-1969 that included 113 cases over a 27 month period, 90,000 people were given passive immunity to the disease during that same time period, almost one third of the local population (2).

TABLE I REPORTED COMMUNICABLE DISEASES IN THE U.S. (1971)

DISEASE	CASES
RUBEOLA	75,290
INFECTIOUS HEPATITIS	59,606
RUBELLA	45,086
DIPHTHERIA	215
POLIOMYELITIS	21
TETANUS	116
TYPHOID	407
PERTUSSIS	3,036
MUMPS	124,939

During the 6-year period, 1963-1968, a net annual distribution of 6.2 to 11.6 million millimeters of all gamma globulins of human origin were reported to the Biologics Surveillance Program by eleven producers in the United States (12). This output constitutes most of the national supply for military and civilian use. Johnson (13) reported that 26 million milliliters of gamma globulin were produced in 1969 with a distribution of only 18 million ml. This surplus was added to the 1,400 kg of lyophilized gamma globulin in storage. It is Johnson's opinion that during the last few years, the pharmaceutical companies have not concerned themselves with trying to increase supplies of gamma globulin. His article (13) is an excellent summary of the causes for fluctuation in the biologicals market.

When a vaccine is extremely effective, even in its impure form, it can almost completely eradicate a contagious disease. Poliomyelitis, for example, afflicted 33,344 in the United States alone in 1953 (24) but only 21 cases were recorded by the U.S. Public Health Service fifteen years later. While this trend decreases the number of doses required annually to immunize against this disease, it also raises the chance that a large percentage of the population has never been immunized against polio. If another outbreak should occur, it would be extremely difficult to protect a large percentage of the exposed population. Annually, large quantities of vaccines are manufactured, stored, and then destroyed when the product shelf life is exceeded if epidemics of the diseases which they protect against do not occur.

SEVERITY ESTIMATE: Affliction by the class of diseases covered in this section can range from a very short period of acute illness with complete recovery, to blindness, permanent cosmetic disfiguration, sterility, and complete paralysis, if not death.

TREATMENT REGIMEN: Improvements and changes are frequently made in the biologicals dispensed by the large drug manufacturers in their attempt to purify each compound. Dosage schedules will therefore frequently change as well. As a guide we will cite the recommended intramuscular doses (11) included in the nineteenth United States Pharmacopaeia (the standard preparation contains about 165 mg gamma globulin per milliliter):

TOTAL GAMMA GLOBULIN DEFICIENCY - initial dose of 1.3 to 2 ml/kg body weight up to 20-30 ml. A maintenance dose of 0.66 to 1 ml/kg up to 20-30 ml once per month.

POLIOMYELITIS - 0.3 to 0.44 ml/kg

RUBELLA - 20 to 30 ml

RUBEOLA - modification of the disease if already afflicted is 0.44 ml/kg. A prophylactic dose is 0.22 ml/kg

VARICELLA - 0.22 to 1.3 ml/kg up to a maximum of 20-30 ml

INFECTIOUS HEPATITIS - 0.022 to 0.11 ml/kg

TETANUS - 250 to 500 units

DIPTHERIA - 20,000 to 200,000 units

TREATMENT METHOD: Vaccination is the method of choice, whenever possible in the long-term prevention of infectious diseases. Vaccines used for prophylaxis create their effect through the stimulation of active immunity. This is manifested in part by the production of protein antibodies in the form of gamma globulin and in part by the stimulation of cell mediated reactions in the recipient. Usually the vaccine is given in three doses to create the active immunity for prophylactic treatment. The first intramuscular or subcutaneous dose sensitizes the cells of the body and the immune mechanisms to the presence of the foreign substance. The second dose given six to eight weeks later will cause a sharp outpouring of protein antibodies in the form of gamma globulin. A third dose is usually given six months after the second injection to produce long-term immunity. Such relatively active immunity commonly lasts for years in a reduced form and can be restimulated by "booster" injections of the immunizing agent.

Active immunization, however, has to be carried out before the individual is exposed to the infectious disease. When a person has already been exposed to one of the above diseases or even after he experiences the onset of symptoms to that disease, he can be given a partial or passive immunity by receiving the specific gamma globulin for that disease from another individual already convalescing or previously afflicted. The protein antibodies thus introduced will give a measure of protection for a period of days or weeks in the ill patient. This immunity cannot be changed to long-term active immunity unless the patient actually contracts the disease.

CURRENT GROUND STATUS: The primary source of gamma globulin is from fractionation of human plasma proteins and extraction from human placentae. The supply of immune globulins has generally been adequate. The American National Red Cross contracts with four or five laboratories to fractionate outdated plasma. Because of the current heavy demand for albumin and plasma-protein-fraction, these laboratories process gamma globulin as a byproduct. It is processed to the dry powdered state by lyophilization and held in storage until the Red Cross requests a particular quantity of 10 ml or 2 ml vials for their stockpile. This is always done from the oldest lyophilized powder on hand. There were 1,400 kgs of surplus lyophilized gamma globulin on hand in 1970. This represents 9,000,000 ml of commercial product. The U.S. currently sells 8 million milliliters overseas annually in an attempt to recover the cost of fractionation. At one time industry had allocated one-third to one-half the costs of plasma procurement to gamma globulin. Since the cheaper and more plentiful plasma-protein-fraction is more in demand as a plasma expander, the economy of plasma component therapy is being threatened.

It is apparent that for the last few years the plasma fractionation industry has not concerned itself with trying to increase supplies of gamma globulin. Their major research emphasis is to provide specific standardized immunoglobulins that are safe, effective, and highly profitable. It is impossible to extract purified antibody for a particular pathogen from this heterogeneous gamma globulin fraction of plasma, but fractions that are enriched in specific antibodies may be prepared from the plasma of individuals convalescing from the disease in question. There are problems with the material fragmenting during storage (and thus losing its potency). Aggregation has been another problem during preparation that must be corrected for ease of processing.

Investigations of antibody formation in man and animals have apparently established the plasma cell as the main source of antibody gamma globulin synthesis. Recent work has supported the concept of lymphocytic and placental origin of some antibodies. Gamma globulin has been detected in these cells using the fluorescent antibody technique.

Gamma globulins have recently been extracted from homogenized human placentae with great success but they contain traces of blood group antigens, making the product undesirable for use with Rh negative recipients.

b) STARTING MATERIAL AND PRODUCTION METHOD: The process is very thoroughly covered by Deutsch (9) and Krijnen and associates (20). Briefly the commercial methods of fractionation of plasma proteins on a large scale have undergone little change in the U.S. over the last 30 years. The standard Cohn fractionation procedure (21) is still the method of choice but with slight modifications discussed below. During the last 10 years normal gamma globulin preparations have been partly replaced in clinical use by specific immune globulins. Whereas normal gamma globulin is isolated from the plasma of nonselected donors, the preparation of specific immune globulins requires the collection of blood either from selected donors with high natural titers against specific antigens or from donors who have been hyperimmunized against specific antigens.

Figures 1 and 2 schematically illustrate the isolation of gamma globulin by the process used in the Central Laboratory of the Netherlands Red Cross Transfusion Services. It is essentially cold precipitation of the various protein fractions using increased concentrations of ethanol under very tight temperature and pH conditions. Fractions II and III are further treated with different concentrations of ethanol, at a more acid pH, colder temperature and specific ionic strength. After the dissolved gamma globulin paste is freeze dried, the protein powder is dissolved in 0.3 M glycine solution containing 0.01 percent thimerosal, to a final protein concentration of 10-16% with a pH of 6.9. Sterile filtration is through asbestos sheets. Electrophoretic quality control of the final product indictated it was 95 percent gamma globulin with an overall yield of about 85 percent. By specifically selecting donors high in a particular gamma globulin fraction, the batch can be processed for immunity against a specific disease.

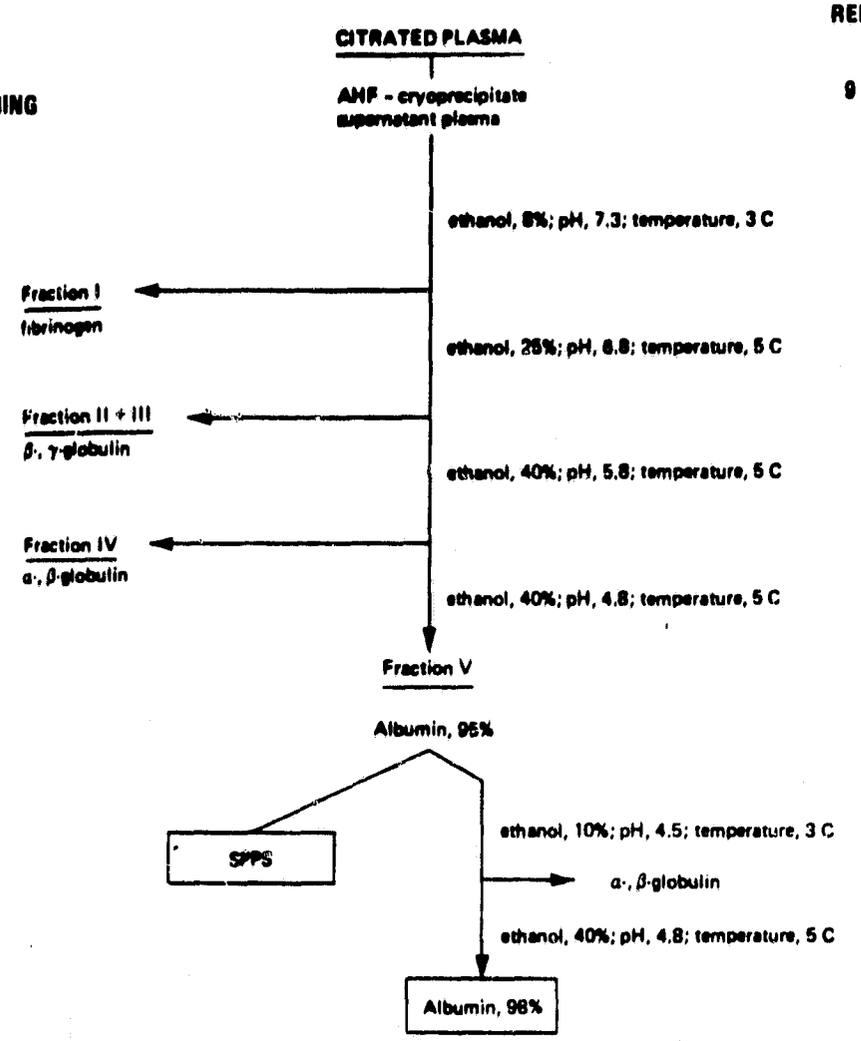

FIGURE 1 Cold-alcohol fractionation method, as used in the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam. SPPS = soluble plasma-protein solution.

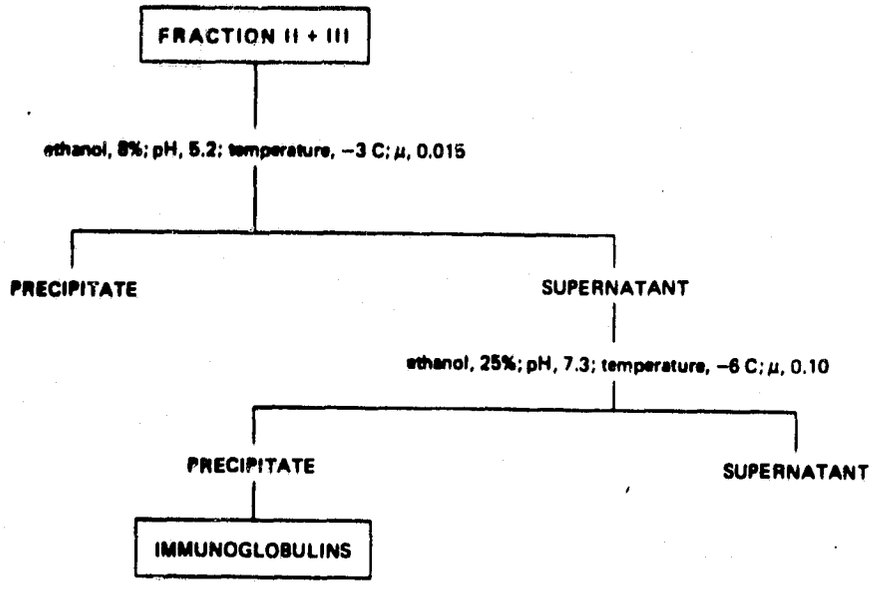

FIGURE 2 Isolation of immune globulin concentrates from fraction II + III.

c) YIELD: The yield of immunoglobulins from one liter of plasma is approximately 4.2 grams or about 85% of the gamma globulin present in the initial plasma protein mixture. More highly purified specific immunoglobulins will reduce both the percentage and weight yields. When gamma globulin is extracted from human placentae, the yield is about 0.5 grams per placenta.

d) PURITY: Gitlin (4) has reported values as high as 95 percent pure nonspecific gamma globul n. When highly specific material is extracted from the original plasma protein batch, there is usually some contaminating protein present. The fragmentation problem with material is believed to be due to traces of proteolytic enzymes. The commercial preparations usually contain glycine as a stabilizer and merthiolate as a preservative. Some brands contain thimerosal, aminoacetic acid and a suitable antimicrobial agents (11). The public health agencies are always concerned about the possible presence of hepatitis B virus being a major contaminant. The gamma globulin of placental tissue sometimes contains Rh antibodies which may cause erythroblastosis.

e) COST/DOSE (to patient) AND AVAILABILITY: The cost of human gamma globulin in a 2 ml vial (about 330 mg) is $2.44 to the physician for private dispensing. A 10 ml vial costs $4.94 and contains 1600 mg. Most dispensing however, is through local, state and federal public health agencies. Because these agencies buy the material in large quantities, discount prices must be in effect. Their purchase price is unknown to us. Until July of 1966 the American Red Cross distributed gamma globulin to all public health agencies free of charge.

The more specific the gamma globulin to a particular disease, the higher the price. Mumps gamma globulin can be as high as $35 per passive immunity treatment. A standard D-P-T polyvalent injection for a child prior to entering school can be as low as $5.00.

The gamma globulins are only available by prescription and can readily be obtained from local pharmaceutical distributors rather than drug stores. Most public health agencies keep a small supply of the material on hand but are not prepared for massive innoculations to fight major epidemics. When this situation arises, the agencies usually borrow or obtain supplies from one another and replace the supplies as soom as possible. At the current time there seems to be an ample supply of gamma globulin in reserve in America but specific immune globulins are in relatively short supply.

Nine American laboratories supply this material. They include Abbott, Armour, Cutter, Dade, Hyland, Lederle, Park-Davis, Pitman-Moore, American Red Cross and Connaught.

f) STORAGE: Gamma globulin can be stored in the lyophilized form. Accelerated degradation tests showed that the material could be stored at -20°C in the lyophilized form for a number of years (23). The preparations usually supplied to physicians are in solution and they readily break down into fragments losing their potency. At 2°C the dissolved material will fragment at about one percent per month. At 20°C this same material will fragment at 7.5 percent per month.

TECHNICAL DATA

a) MOLECULAR WEIGHT: The gamma globulins represent about 13 to 23 percent of the total plasma proteins. Most of the antibodies found in plasma are associated with the gamma globulin fraction although a minor portion is found in the Beta globulin fraction. Globulin is a heterogenous mixture comprised of a number of different immunoglobulins. At least 30 different subfractions have been prepared from human proteins to date. These subfractions vary widely in molecular weight. Recent work with ultracentrifugation techniques has classified globulins into three major subfractions and several minor subfractions. Most of the antibodies are in the immunoglobulin G fraction (IgG) which represents about 70% of the total gamma globulins. This fraction has a molecular weight of about 150,000 and contains 2-3 percent carbohydrate. The IgA fraction has a molecular weight of 180,000 to 500,000. The IgM or macroglobulin fraction has a molecular weight of approximately 1,000,000 and a carbohydrate content of about 10 percent. Upon treatment with thiol reagents, macroglobulin splits into five subunits each having a molecular weight of about 185,000. Additional gamma globulin fractions are known but are currently of little import.

b) MOLECULAR STRUCTURE AND AMINO ACID SEQUENCE: The immunoglobulins are glycoproteins containing 2-12% carbohydrate including mannose, galactose, fucose, glucosamine and sialic acid. They apparently differ only in minor ways with respect to amino acid composition.

The different types of immunoglobulins are made up of subunit chains. Two general sizes of chains have been found; the A or heavy chain has a molecular weight of

about 40,000 while the B or light chain has a molecular weight of 20,000. Further breakdown of the A and B chains has been accomplished according to their origin in the electrophoretic separation. The complete amino acid sequences of several human types IgG and IgM and about half of that of an IgA has been determined. The heavy chains of each contain a 330 amino acid residue sequence that is common to all the immunoglobulins and a variable 110 amino acid sequence that is specific for each. The light chains contain about a 50 percent mixture of constant sequence and variable sequence portions of each chain. Disulphide linkages are present to connect the chains and peptide subunits. Full reconstruction of the immunoglobulin's three dimension configuration has not been established but it is believed to be coiled into an ellipsoid. A more detailed review of these proteins can be found in Putnam (5) and Orten and Neuhaus (6).

c) ELECTROPHORETIC MOBILITY: Plasma proteins have been subjected to a variety of electrophoretic techniques. Gitlin (4) described some of the earlier work using filter paper, cellulose acetate, starch, agar and Tiselius moving boundary techniques. Many papers in the book edited by Putnam (5) describe electrophoretic techniques used to obtain separation. In general, the gamma globulin fraction is the slowest migrating fraction of all the plasma proteins moving toward the anode. Electrophoresis is currently the method of choice for clinical analysis of changes in gamma globulin.

d) ISOELECTRIC POINT: Between pH = 5.6 and pH = 5.9 for γ_1 globulins and between pH 7.3 and 7.6 for γ_2 globulins (4).

e) SOLUBILITY: The immunoglobulins are usually soluble in 15-18 percent solutions of sterile distilled water or physiological saline. The material is kept in solution at a pH of 6.4 and stored at 4°C. If the temperature is elevated or the pH is raised to 7.2 the material will fragment to smaller nonfunctional peptides (7).

Painter (8) found gamma globulin stored at 2°C fragmented at a rate of about 1% per month. When the same material was stored at 20°C, the fragmentation rate was about 7.5% per month. This fragmentation is believed to be due to the presence of proteolytic enzymes in the partially purified immunoglobulin.

The commercial preparations contain glycine as a stabilizer and merthiolate as a preservative. Some preparations contain aminoacetic acid and a suitable antimicrobial agent (11). The material is insoluble in cold ethanol but can go back into solution upon rewarming to body temperature. A departure from the usual fractionation procedures has been the introduction of a method that uses Rivanol (2 - ethoxy-6, 9-diamino acridine) in conjunction with ammonium sulfate (9). The use of Rivanol is based on its ability to form relatively insoluble complexes with proteins in their anion form. In contradistinction to the ethanol fractionation procedure, the first precipitation in this method removes proteins of lower isoelectric point and leaves IgG in solution. This procedure is currently being used by the Behringwerke in Marburg, Germany (10).

f) SPECIFIC GRAVITY: The specific gravity of plasma cells is not known to us.

g) TISSUE CULTURE: Antibody production is known to be a function primarily of a class of lymphoid cells known as plasma cells, and, to some extent, of their precursor cells, the B-lymphocytes. Manufacture of the antibodies occurs in peripheral blood and in tissues where these cells are concentrated, such as the placenta and the walls of the gastrointestinal tract. In addition, there is some evidence that macrophages may participate in antibody production, although they probably do not play a major role. Because human placentae are so easily attainable as a source of gamma globulin, tissue culture of plasma cells and lymphocytes has not been pursued with that purpose in mind. (In 1969 there were 3.4 million births in the U.S. and 70% of the placentae were used for the production of gamma globulin.)

h) METHOD OF TISSUE CULTURE: Most standard textbooks on tissue culture will give details on lymphocyte culture methods. Paul (15) describes monolayer culture in detail. Suspension culture techniques are described by Lazarus and Foley (16). Tissue culture methods for antibody production in vitro are covered in the book by Williams and Chase (22).

i) TECHNIQUE OF ASSAY: The standard technique for identification and quantitative analyses of specific immunoglobulin is immunoelectrophoresis. This is an extremely sensitive and useful technique combining electrophoretic separation with the specificity of immunologic reactions. The proteins are separated as a series of spots or bands on an agar covered glass plate by the usual electrophoresis procedure. The current is turned off and the proteins diffuse outward. Simultaneously to

this diffusion, an antihuman immune serum protein from another animal species which contains antibodies for the protein under investigation is placed in a trough at the side of the agar plate but parallel to the path of electrophoretic migration. The antibody diffuses inward and when it meets the specific antigen, the familiar precipitation occurs, forming an opaque arc.

EXPECTED SPACE IMPROVEMENT: Processing of this material in space could offer a higher level of purity for the specific gamma globulins. Because of the relatively inexpensive high yield processing methods used on earth and the abundant quantity of material in storage, space probably can not compete favorably with regard to production and economics of general gamma globulins except as a byproduct from other plasma fractionations.

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NAME (GENERIC): Pancreatic Beta Cells
(PROPRIETARY): None

DISEASE TREATED: Juvenile Onset or Insulin Dependent Diabetes Mellitus

PATIENTS: About three million people in the United States have juvenile onset type diabetes (1). There is considerable confusion about the actual number of patients suffering from diabetes mellitus. The leading manufacturer of Insulin, Eli Lilly and Company, estimates about 1.2 million people require treatment for diabetes with insulin (3). This same company estimates there may be as many as 10 million American people who are either unidentified diabetics or being treated with controlled diets and synthetic pharmacological agents for the milder form of the disease (i.e., patients that develop diabetic symptoms after age thirty). This possible patient population is also quoted in the latest edition of the most commonly used text book on Clinical Endocrinology (5). The National Center for Health Statistics conducted a health interview survey in 1973 (4) showing that 13 percent of the population over the age of 17 was suffering from some form of diabetes mellitus. Of these 26,000,000 people, half are over sixty-five years of age and one-third have limitations to their activities. There are more men than women diabetics. This disease is prevalent among certain ethnic groups, e.g., Jewish, Pima Indians (50% afflicted) but it is not limited to any specific national origin or genetic population. The National Commission on aging reported the number of diabetics in the United States increased by 50 percent during the period between 1965 and 1973.

Diabetes is the fifth leading health related cause of death. More than fifty percent of diabetics die because of coronary disease. Renal failure is the cause of death of most juvenile onset diabetics. Diabetes frequently causes cerebral vascular disorders, gangrene of the legs and neuropathy. It is the second commonest cause of blindness. The total annual costs for the disease in the United States are estimated to be at least five billion dollars including medical care and the loss of compensation for work.

The number of diabetics in the world is unknown. The disease was noted in written records as early at 1500 B.C. in the middle east. In Great Britain it is estimated

that four percent of the population is affected by diabetes mellitus. The Eli Lilly Company has processing plants around the world to make pancreatic extracts of insulin.

SEVERITY ESTIMATE: Diabetes mellitus is considered a severe chronic disease. Cessation of medication can result in coma and death. Inadequate medication can result in chronic wasting and vascular disease causing blindness and the necessitated amputation of appendages.

TREATMENT REGIMEN: In inbred laboratory animals, a single injection of 600-800 islets of Langerhans (containing beta cells) directly into the hepatic portal vein will reduce and possibly eliminate the need for supplemental insulin injections (7).

TREATMENT METHOD: Beta cells are found in the islets of Langerhans located in the pancreas. These cells produce a hormone called insulin that is responsible for lowering glucose in the blood by aiding in its absorption into the cells for metabolic activity. If these cells are absent from the body or only available in limited supply the individual will have a disease known as diabetes mellitus. For all but the mild cases, insulin injection is a specific and only method of treatment. Of all the endocrine therapies, this has saved more lives than any other.

An experimental method of treating diabetes for the last several years has been investigated by laboratory and supporting clinical research teams at Washington University Medical School, Mayo Clinic Graduate School of Medicine and the University of Minnesota Health Sciences Center (7,28,26,27,28,29,8). These studies have indicated that isolated islets of Langerhans can be transplanted successfully in animals and diabetic symptoms supressed (25,26). Human pancreatic islets have been isolated from cadavers (27,28) and transplanted intramuscularly, intraperitoneally or into the portal vein of seven patients suffering from endstage diabetic nephropathy (8). Najarian and associates observed a transient reduction in insulin requirements lasting two weeks to two months in six of the patients. None of the patients were cured of diabetes and all transplanted islets eventually died because of immunological rejection. The investigators felt that a larger number of beta cells or islets must be transplanted to be effective and there may be a requirement for donor-recipient tissue matching as currently used for kidney and heart transplants.

The treatment of choice for juvenile onset diabetes mellitus over the last fifty years has been the self administered subcutaneous injection of 10-20 international units of insulin 3-4 times a day for the rest of the patients' life (9). This procedure has many undesirable complications not the least of which are: a) determining the correct variable dosage for each injection to prevent over-dosage and hypoglycemic shock, b) the rotation of injection site to prevent skin necrosis and "sloughing," c) the sterility requirements of syringes, needles and insulin, and d) the inconvenience and discomfort of the life long injections and carrying the medical equipment on your person at all times. (In some states possession of a syringe, even when jointly found with insulin but not co-located with a doctors prescription, can lead to the inconvenience of police questioning and possible arrest.)

Pharmaceutical companies have developed modifications of the insulin extract to reduce the number of injections required by the diabetic to one per day. The use of protamine zinc, globulin, isophane, lente and ultralente forms of insulin have been helpful to many diabetics but are unacceptable to some because of allergic reactions.

Those people developing diabetes later in life as the result of aging or "sluggishness" of the pancreas start treatment with dietary control. Oral hypoglycemic drugs (such as Diabinese), which are believed to stimulate the release of insulin from the pancreas, are sometimes used as adjunct therapy, although their safety has recently come into question. Finally, as a last resort, they may begin insulin therapy similar to juvenile onset patients. Although Diabinese alone has controlled some maturity onset diabetics during the stress of mild infection or minor surgery, insulin is essential during intercurrent complications (44).

CURRENT GROUND STATUS: The status of pancreatic transplantation (total organ and cell components) for treatment of diabetes has been reviewed by Matas (8) and a position paper presented by the American Diabetes Association (31). Initial attempts to transplant adult pancreatic fragments were unsuccessful because the associated exocrine enzymes autodigested the transplanted tissue or injured the host (32). Research emphasis was directed to isolation of the active insulin producing islets for transplantation (25, 26). A major contribution to the field of islet transplantation was made by Kemp and associates (33) when they demonstrated that implantation of islet cells via the portal vein was more effective than intraperitoneal

transplantation. Matas (34) found that specific culture conditions resulted in the depletion of digestive enzymes within the pancreatic enzyme tissue but allowed islet tissue to survive. Preservation of islet tissue is another major problem to be solved before clinical application of islet transplantation can be wide spread. Frankel and associates (35) have been able to store isolated mouse islets in medium 199 at 8°C for up to five weeks with retention of the ability to release insulin in response to glucose stimulation.

Strautz (21) has studied the effects of implanting isolated pancreatic islets contained in Millipore filter capsules into hereditary obese mice. These transplants worked for the 45 days duration of the test; the experiment objective was not related to length of islet exposure. The method of islet isolation was described by Kostianovsky and Lacy (20, 22).

Successful islet transplantations in diabetic animals have been reported (7, 25, 26, 29) but only one study of partial success in seven human patients has been conducted (8). Ballinger (26) and Ashcroft (27) were able to isolate islets from the pancreas of a child who underwent subtotal pancreatectomy for functional islet cell hyperplasia. These cells were functional in vitro but resulted in a yield of from 1-50 percent of the total islets in the original pancreatic material. The average yield was closer to 5 percent by their methods. These investigators feel that the collagenase digestion-Ficoll gradient technique will not provide a sufficient quantity of islets for human islet transplantation to be consistently feasible, and other approaches for islet preparation will be needed.

The beta cells have recently been replicated in monolayer culture in the laboratory but the process has not continued to the extent of making the process commercially feasible on the ground (48). Hellman has reviewed several methods for the microdissection of pancreatic islets away from acinar tissue and rapid ways for estimating the number and sizes of these cells in a whole pancreas (19). There is no current commercial preparation of beta cells. An experimental method is currently under investigation as described in Treatment Method.

The primary source for insulin to treat diabetics is the pancreatic tissue of cattle and pigs. The Eli Lilly Company processes 13,000 kg of animal pancreas per week in their Indianapolis plant. Each pancreas is received in the frozen state from

one of the commercial slaughter houses throughout the country. Then, remaining in the thawed but refrigerated state, it is trimmed of fat, homogenized, defatted and insulin extracted by a proprietary method. Commercial insulin is 95 percent pure. The 5 percent contamination consists partially of insulin polymers, pro-insulin and degradation products. There has been continued improvement in purifying the product, with great progress made recently by using sephadex chromatography to exclude the larger molecular weight aggregates. This more pure material (99 percent) is now termed "single peak" insulin and is available for use. Williams (5) expects a "single component" insulin, 100 percent insulin-molecular weight 6000, to be commercially available within the near future. The average yield of insulin extract per pancreas is approximately 0.002% of the wet tissue weight. The insulin is then combined with zinc, protamines or other proteins to reduce its rate of release in the human body. The finished but not completely purified product is prepared in an aqueous concentration of either 2, 4 or 5 mg/ml (24 units/mg). To this colorless sterile solution is added 0.1% (w/v) of phenol or 0.25% (w/v) of cresol as a preservative. The final marketed product also contains between 1.4% and 1.8% (w/v) of glycerin (9).

Insulin, as usually prepared, has 24 units/mg, and then 1 unit equals 41 micrograms of insulin. The unit is an arbitrary quantity, determined usually by bioassay. Although bioassays are no longer widely used, commercial preparations of insulin are still standardized in terms of this arbitrary unit. Physicians still prescribe insulin in terms of units of insulin and radioimmunoassy laboratory reports are recorded in insulin units.

It is very difficult to extract insulin from the pancreas owing to rapid destruction of all proteins by the proteolytic enzymes released from the acinar cells surrounding the islets of Langerhans. Because of this, all processing is done at close to freezing temperatures (2-7°C) to minimize chemical reactions.

Each bottle of insulin produced in the above manner represents one animal. The pharmaceutical companies are concerned that an increasing world population will require an increased production of insulin to meet the anticipated projection of new diabetics. These companies feel that they have nearly reached the limit of available animal pancreatic material and therefore are interested in any new sources

of supply. The availability of insulin for treatment of diabetes is compounded by religious factors as well. Certain religious groups, e.g., the Jewish and Moslem faiths and some middle eastern churches, are forbidden to consume pork products. Therefore, they are limited to insulin prepared from cattle. While the protein structure of insulin is similar to human insulin from all three species of animals now used as the source of supply, allergic or resistent reactions have developed in a number of patients. This requires switching animal sources for the allergic patients. Ideally they should be receiving human insulin but this would necessitate collecting the pancreases of approximately 200 deceased donors/year/diabetic!!

b) STARTING MATERIAL FOR PRODUCTION OF BETA CELLS: The human pancreas has one to two million islets of Langerhans (1). They are scattered among the acinar cells of the pancreas. These acinar cells contain proteolytic enzymes which will rapidly destroy insulin if they come together. The small size of the islets (20-300 microns in diameter) coupled with their scattered location have discouraged many cellular observations.

Each islet of Langerhans contains three types of cells, each producing a hormone. The alpha cells produce glucagon, a hormone that raises blood sugar; the beta cells produce insulin which lowers blood sugar and may play an active role in transporting nutrients across cell membranes, and the delta cells produce somatostatin, which inhibits release of insulin and glucagon under some circumstances (45). Between 60-80 percent of the islet cells are beta cells. Each beta cell contains about 1.7 microunits of insulin and the entire human pancreas about 200 units at any one time indicating about 120 beta cells per islet of Langerhans (1). Each islet is surrounded by a basement membrane. There is an elaborate labyrinth of anastomosing capillaries in each islet.

The current method of maintaining islet cells is to sustain them in monolayer tissue culture (11,12,13,14,15). The technique of cell culture will be presented below. Braaten and associates (11) have found that the addition of heavy metals (sodium ethylmercuriothiosalicylate, phenyl mercuric acetate, phenyl mercuric nitrate and sodium aurothiomalate) to the culture media selectively destroys the contaminating fibroblastoid cells yielding highly enriched, morphologically intact, functionally competent endocrine cells that are capable of cell replication. They also found that fetal cells were more sensitive to fibroblast toxicity than neonatal cells.

c) YIELD: Each human pancreas contains about one million islets of Langerhams. According to Matas (46), the minimum quantity of islet tissue required to cure human diabetes is approximately six percent of the islet mass present in the normal adult pancreas, or about 60,000 islets. Najarian (47) sets the estimate even higher, stating that 20 percent of adult islet cell mass will be needed to cure diabetes by transplantation; this would require 200,000 living islets for each diabetic patient treated. Since the recovery of islets by traditionally used isolation methods is usually in the 5-10 percent range, it is unlikely that sufficient islets to constitute one transplant could be obtained from one adult donor. However, infant and fetal pancreati, which contain a much higher ratio of islet cells to exocrine acinar cells than that of adult pancreati are believed to be a more promising source of transplantable islet tissue.

d) PURITY: Electrophoretic separation and possible clonal cell culturing should provide an exceptionally pure strain of beta cells.

e) COST/DOSE (to patient) AND AVAILABILITY: Islet cell transplantations to date have been done as experimental research. These cells are not available for pharmacological purposes. In view of this situation, a cost per person for the anticipated treatment dose of islet cells has not been established and awaits further technological development and market analysis. Since the average insulin dependent diabetic will pay about $10,000 (in 1977 dollars) for the currently available insulin over his life time plus the cost of the injection and sterilization paraphenalia, the cost of a single injection of islet cells could be very expensive but still within the acceptable market.

f) STORAGE: These cells can be stored in the frozen state. Cryoprotectants may have to be added to preserve the cells going through the rate-freezing process. Since these cells continue to metabolize in the frozen state, even though at an extremely low rate, the cells will have a definite storage life expectancy. This storage life still has to be determined.

TECHNICAL DATA

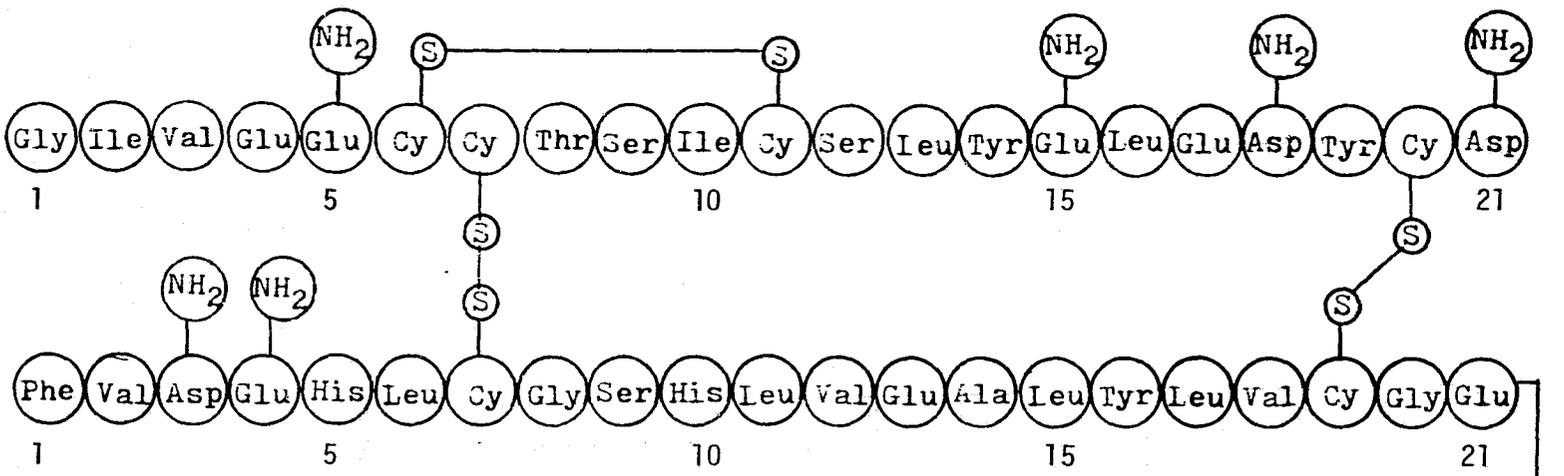
a) MOLECULAR WEIGHT: 6000 (insulin)

b) MOLECULAR STRUCTURE AND AMINO ACID SEQUENCE: Insulin consists of two peptide chains, the A chain and the B chain connected by two disulfide bridges (1). The A chain has 21 amino acids, is acidic and has an N-terminal glycine. The B chain has 30 amino acids, is basic and has a N-terminal phenylalanine. Insulin is formed from a single 84 amino acid molecule (pro-insulin) folding upon itself and having both the A and B chains (each end of the molecule) joined by two disulfide bonds. An enzyme breaks out the central 33 amino acids (C chain) to leave the active insulin molecule. Usually 0.4% zinc is present.

The amino acid composition and molecular weight of insulin differs from species to species. There is, however, enough similarity between human, bovine, ovine and porcine insulin that these forms can all be used in human medication. Side effects may occur because of this species difference but it is not common. There can be a difference in as many as 23 of the 51 amino acids, as long as disulfide bonds are present, without much change in endocrine activity. Five or six amino acids can be removed from the B chain and still have some activity. The amino acid sequence for human insulin is shown in Figure 1. It was the first protein so identified. It has been synthesized in the laboratory (2) but the process is too complex for commercialization. The synthetic process gives very poor yields (usually less than 10%) and is not anticipated as a manufacutring process threat to current insulin extraction methods.

The secondary and tertiary structure of insulin are probably important in the hormone's action and antibody formation but are not well known or understood at present. Hodgkin published a three dimension structure for the molecule but it has not been confirmed (6). Two atoms of zinc were found to be present with six molecules of insulin in a spheroid unit. The A chain rested in a pocket made by three main sections of the B chain. The surface of the molecule exposed the polar groups together with a few non-polar residues.

c) ELECTROPHORETIC MOBILITY: Beta cells and islet of Langerhans mobility is unknown. "Purified" insulin migrates electrophoretically to the cathode, but increasingly strong treatment with acid extraction techniques (removing more amino groups) will eventually cause it to migrate to the anode (5).



	Chain A			Chain B
	8	9	10	30
Man	Thr	Ser	Ile	Thr
Pig	Thr	Ser	Ile	Ala
Rabbit	Thr	Ser	Ile	Ser
Beef	Ala	Ser	Val	Ala

Figure 1. Structure of Human Insulin and Differences with Other Species

Electrophysiological studies have shown that the beta cells from mouse pancreatic islets have a cellular transmembrane potential of -20.1mV and the resting potential can be affected by exposure to glucose, mannose, leucine and tolbutamide. These substances which are also known to evoke insulin secretion induce small action potentials in the islet cells (16). Changes in the composition of the extracellular fluid can also influence the electrical characteristics of the beta cell action potentials (18). A solution of 5mM Alloxan depolarized islet cells but not the acinar cells of mouse pancreatic segments. Dean and Matthews (17) review a number of compounds that may modify the electrical surface potential of the cell and thus its electrophoretic mobility.

d) ISOELECTRIC POINT: 5.30 - 5.35 (Insulin). It has not been determined for islets of Langerhans or beta cells to our knowlege.

e) SOLUBILITY: Beta cells and islets of Langerhans are insoluble substances.

Insulin is readily soluble in dilute acids and alkalis. In an acid medium it will exist as a dimer but in 30% acetic acid it will be in monomeric form. It is insoluble in the pH range of 4.5-7.0. Molecular dissociation is favored by extremes of pH and very low ionic strength. Isolated islets will release large quantities of insulin when suspended in Tris (hydroxy methyl) aminomethane buffer in a pH range of 7.4-9.0 (24).

Insulin readily adsorbs loosely to many substances including cellulose, agar, glassware, and certain serum proteins. Many proteins inhibit adsorption but albumin and casein are two of the better compounds for preventing this (5).

f) SPECIFIC GRAVITY: Islets of Langerhans apparently have a specific gravity in the range of 1.045 - 1.085.

g) TISSUE CULTURE: The current method of providing islets cells is to maintain them in monolayer tissue culture (11, 12, 13, 14, 15). One problem in obtaining functional cultures was overcome by the work of Braaten and associates (11) when they found a method of eliminating the fibroblast membrane that encapsulated the culture and virtually halted cell replication. However, cultured islets do not proliferate; rather they undergo a steady decline in mass and number with the

passage of time. Frankel and associated have been able to store isolated mouse islets in Medium 199 at 8°C for up to five weeks with retention of insulin production and function (35). Islets stored in culture are slower to respond (36) when transplanted into a host when compared to newly isolated cells (less than two hours out of the donor). However, the culturing procedure offers considerable flexibility for selection of recipients and method of implantation.

h) METHOD OF CELL CULTURE: Animal pancreatic tissue was cultured by the method of Sharp (10). Usually fetal tissue was used. Enzymatic disruption of the finely minced pancreas was accomplished by adding collagenase (90% type IV and 10% type I) to a 7 mg/ml tissue suspension in calcium and magnesium free phosphate buffered saline in a stoppered test tube that was vigorously shaken in a 37°C water bath for 10-12 minutes. The mixture was transfered to a 25 ml graduate cylinder in ice to stop the enzyme reaction and then the suspension was washed three times in ice cold base medium with 5 minute sedimentation at unit gravity at each time. The final sediment was left in a volume of approximately 3 ml and suspended at room temperature in a solution of FICOLL 400 diluted with 199 base culture medium with unmodified Earl's salts to a final volume of 30 ml and a density of 1.100 in a 50 ml plastic centrifuge tube. A discontinuous gradient was then formed by layering 5 ml of diluted FICOLL at densities of 1.085, 1.075 and 1.045 above the tissue suspension. The gradient was centrifuged at 800"g" for 30 minutes in a swinging basket rotor. The islets of Langerhans floated mainly to the interface between 1.045 and 1.075 as well as 1.075 and 1.085 from which they were harvested with a 14 guage needle; acinar and non-endocrine pancreatic tissue remained in the cell pellet. The harvested islets were washed with culture medium, centrifuged for 10 minutes at 150"G" and seeded in 35 mm plastic monolayer culture dishes at a density equivalent to one pancreas per dish. The culture medium 199 with unmodified Earl's salts and glutamine, 10% fetal calf serum, glucose 3 mg/ml, aqueous sodium penicillin 400 units/ml and amphotericin B 1.5 µ/ml was changed every 48 hours. The culture dishes were incubated at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide. The thimerosal of Braaten (11) in a concentration of 2.8 micro moles/ml must be added to the culture 24 hours after seeding or confluent fibroblasts will envelope the monolayer by 3-5 days inhibiting further endocrine cell replication. The dose of thimerosal is dependent on the age of the donor tissue, and the density of the cultured cells. This thimerosal must be added to each medium change to hold down (but not stop) production of fibroblast cells.

i) ASSAY TECHNIQUE: The islets of Langerhans and beta cells can be identified visually by light microscopy if proper staining is used. Maximow and Bloom (37) reported the use of neutral red and Janus Green differential staining of the beta cells. These stains are toxic to the cells, however. Additional work will be required to develop techniques for differentiation of viable beta cells and/or islets of Langerhans from the surrounding tissue.

Insulin can be detected by a number of methods. The original technique was based on the effect of insulin on the blood sugar of the rabbit. Early preparations were standardized by finding the amount required to lower the blood sugar of a normal 2 kg rabbit to 45 mg per 100 ml within a five-hour period (38). Biological variation is considerable and requires special statistical design. Greater sensitivity can be obtained by using hypophysectomized, adrenalectomized or alloxan treated rats or mice as test preparations. When insulin is injected into these animals it is possible to detect between 0.05 and 0.5 μ units (39). In vitro methods of assay using liver slices (40) rat diaphragm (41) and adipose tissue have been reported (42). These in vitro techniques test the ability of the unknown sample to increase the uptake or oxidation of radioactive glucose. Immunological assays, especially radioimmunoassays have been in existence for about fifteen years (43). They are very sensitive and can detect as little as 1 picogram of insulin (0.025 microunits).

EXPECTED SPACE IMPROVEMENTS: The electrophoretic separation of islets of Langerhans in a zero-gravity environment is expected to yield a greater number of viable cells in a shorter period of time than current ground separation techniques. The method of separation may yield a more pure injectable product by removing some of the extraneous cell debris that is currently a problem in preliminary clinical trials.

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NAME (GENERIC): Somatomedin, Sulfation Factor

(PROPRIETARY): None

COMMERCIAL USE: Promote Livestock Growth in Weight and Size

COMMERCIAL USAGE REQUIREMENTS: Over 42,856,000 cattle, 77,303,000 pigs, and 6,355,000 sheep were slaughtered in the United States in 1977. Their total aggregate live weight was almost 62 billion pounds. It now takes about ten pounds of botanical material to add one pound of weight on a herbivorous animal. A number of agricultural biologists believe that a more efficient utilization of the food chain could maintain or expand protein food supplies throughout the world in the presence of a continuously expanding population. If a method could be developed for better animal utilization of nutrients in the livestock food crops, the amount of meat available for market would increase.

During our visits to various pharmaceutical companies, it was suggested that somatomedin may be of use for this purpose. This hormone stimulates nucleic acid and protein synthesis in skeletal tissue (22) and lipid synthesis in fat cells (7). Pharmacological research was being conducted by these companies in an attempt to determine biological efficacy and dosage. Because the dosage information was proprietary, we have arbitrarily chosen one half the dose of growth hormone or one unit (0.05 mg)/40 kg of body weight given three times per week until the animal reaches maturity. This value would provide a comparison dosage with other candidate products in this report. It is known that normal human somatomedin serum levels are about one thousand fold that of human growth hormone (7). Fryklund reports, however, that pharmacological effects were seen in vivo in rats at ten times the normal growth hormone concentration. They did not think that was the optimal dosage. Lund-Larsen and associates found that the relative increase in sulfation activity with increasing serum concentration is the same for human serum and bovine serum (23). The normal concentration of somatomedin in cattle below the age of six months is very low, less than humans, and rises very sharply after that.

In addition to its use in animal husbandry, somatomedin would be of great value in human therapeutics. Pituitary growth hormone is believed to act as a stimulator of somatomedin secretion; therefore, any condition treatable by growth hormone (i.e., dwarfism, stress ulcers, osteoporosis, and nonhealing fractures) would also be treatable by somatomedin administration (27).

CURRENT GROUND STATUS: Although growth hormone is generally regarded as the principal hormone regulating skeletal growth, evidence has accumulated that growth hormone by itself does not stimulate linear growth directly but induces the formation of secondary growth promoting factors. Since the discovery by Salmon and Daughaday in 1957 that serum contained a growth hormone dependent activity (14) several such factors have been purified. Different purification procedures and bioassays have led to at least four different polypeptides being purified from human and animal plasma; somatomedins A, B, C and nonsuppressable insulin-like activity (NSILA-S). Somatomedin A designates the factor which stimulates incorporation of sulfate into chick cartilage, somatomedin B the factor which increases DNA synthesis in glial cells and somatomedin C the factor enhancing both sulfate and thymidine uptake into rat cartilage. These hormones work on a number of tissues in the body causing both an increase in weight and protein synthesis.

The somatomedins are found in very minute traces in the blood and urine. The normal concentration in humans is about 20 micrograms/ml of plasma. By the current methods of processing outdated plasma, less than 0.5 mg quantities of the somatomedins have been obtained for biochemical analysis, characterization and the development of diagnostic tests. This lack of material has prevented any further investigation into its physiological and pharmacological effects in both humans and animals. While there is an absence of such information in the literature, it is known that a veterinary pharmaceutical company is vigorously pursuing such activity. The physiological effects of both an excess and shortage of somatomedins on human growth has been reported in the literature (24, 25, 26).

b) STARTING MATERIAL, PRODUCTION AND FINISHED PRODUCT: Human plasma is the main source for obtaining somatomedins. Fractionation of outdated plasma on an industrial scale is accomplished by a modification of the Cohn method VI (19). Fraction IVb, in lots equivalent to 1000 liters of starting plasma, is extracted several times with acid-ethanol at 5°C. When this extract is then neutralized, additional precipitate is formed which can be removed by centrifugation. The proteins in the supernatant, together with large quantities of salt, are then precipitated using a mixture of acetone and alcohol at -15°C. This extract, when dried, weighs about 85-250 gms, depending on the salt concentration, and contains approximately 30 gms of protein.

This dry acetone: alcohol extract is dissolved in twenty percent formic acid leaving a large insoluble residue. The soluble portion is chromatographed on a large (10 x 90 cm) column of Sephadex G-75. When using this size column, the somatomedin activity can usually be recovered between Kd 0.54 and 0.85. Since several batches are required to process the extract derived from 1000 liters of plasma, the active fractions are pooled and then passed through a Sephadex G-50 column using 0.02 N hydrochloric acid as a carrier.

The active fractions are then subjected to electrophoretic separation. Van Wyk and associates (1) used isoelectric focusing in two steps. The first step covered a broad range between pH = 3 and pH = 10. They used an LKB ampholyne column with a 2 percent ampholyte mixture in 6 M urea and sucrose gradients. The anode buffer was 0.13 M H_3PO_4 in sucrose and the cathode buffer was 0.3 M ethylene diamine in water. Focusing was carried out at 5° C for 87 hours at approximately 300 volts potential difference between the electrodes. The active fractions were collected and resubjected to a second isoelectric focusing procedure but over a narrower range of pH, ie - 2.5, 6-8, and 8-10 - depending on the type of somatomedin they wanted to recover. Final purification was accomplished using several additional Sephadex chromatographic steps followed by another electrophoretic separation in a polyacrylamide gel (15%) at a pH of 2.3 as well as a last separation through Sephadex G-50. A total of 243 micrograms of somatomedin C were recovered from 1200 liters of plasma.

Rather than isoelectric focusing, Fryklund and her associates have used cellulose column electrophoresis in several stages (7). After electrophoretic separation using a 2 x 100 cm column with 0.05 m N-ethyl morpholine acetate buffer at a pH = 7.5, the active fractions were added to a second electrophoretic column (1 x 100 cm) with 0.05 M pyridine-acetate buffer at pH = 50. Those fractions showing activity were then passed through a Sepahdex G-50 column (1.6 x 93 cm) and eluted with 0.02 N hydrochloric acid. A final separation was accomplished on a cellulose electrophoresis column (0.75 x 50 cm) containing 7.8% ethyl alcohol and 2.5% formic acid at a pH = 2.0.

Using iodinated standards to determine the distribution of somatomedins through the various processing steps,it was found that only 0.6 mg of somatomedin A and 2 mg of somatomedir B were extracted from 100 gms of protein in a Cohn fraction IV

equivalent to 1 ton of plasma. This amount of somatomedin A had a specific activity of 3600 units/mg. One unit is equivalent to the amount of somatomedin in one ml of plasma. The Fryklund group reported that many of the problems encountered during the isolation procedure, such as low yields, loss of biological activity and the spreading of activity into several fractions, can possibly be accounted for by the degradation of the parent molecule. They believe the ideal extraction procedure should probably involve a specific adsorption step using antibodies.

c) YIELD: The yield obtained by the procedures of Fryklund and associates (7) was 0.36% of the original somatomedin found in plasma protein fraction IV. Van Wyk and associates (1) had a yield of only 243 micrograms of somatomedin C from 1200 liters of starting plasma using their process which included isoelectric focusing.

d) PURITY: The material isolated by both the Fryklund and Van Wyk groups is reported to provide single bands when analyzed by electrophoresis and gel chromatography. Both groups, however, have reported the presence of breakdown products in their final materials resulting in a heterogeneity.

e) COST/DOSE (to patient) AND AVAILABILITY: The somatomedins are not available commercially for clinical or research purposes. Almost all the material produced for research is done in academic laboratories with the exception of that done in the AB Kabi Laboratories in Stockholm. It is not expected to be available for clinical trials or commercially for the next several years.

TECHNICAL DATA

a) MOLECULAR WEIGHT: At the present time there are three types of somatomedin, each with its own molecular weight and possibly different chemical composition. Somatomedins A and C have an approximate molecular weight of 7000 daltons (1, 18). Somatomedin B is in the molecular weight range of 5000 daltons (2). When whole plasma was separated either by gel chromatography or ultrafiltration, somatomedins A and C, as determined by bioassy, were mainly recovered in a molecular weight range above 50,000. Under dissociating conditions, such as acid-ethanol extraction, somatomedins A and C appeared in a lower molecular weight range leading to the assumption that this hormone was bound to a carrier molecule (3, 4).

b) MOLECULAR STRUCTURE AND AMINO ACID SEQUENCE: Somatomedins are small polypeptides. The amino acid composition of somatomedins A and B have been determined by Fryklund and her associates (5) and are shown in Table I. These somatomedins are very different from one another in that, of the 60 amino acid residues found in somatomedin A and 44 found in somatomedin B, only aspartic acid and serine are found in both hormones in equal quantities. Leucine, isoleucine, histidine and tryptophan are found in somatomedin A but not in B. End terminal analysis by both the Edman degradation procedure and carboxypeptidase A showed that residues 1 and 2 in somatomedin B are aspartic-glycine and residues 43 and 44 are valine-threonine (6). The N terminal group on somatomedin A is Asparigine (7). Niall, as reported by Van Wyk (1), has done a preliminary amino acid analysis on somatomedin C. It is an arginine rich peptide of about fifty odd residues, exclusive of tryptophan or cystine which were not measured.

Fryklund and associates (7) have found several preparations of somatomedin A and B which showed varying degrees of activity. Although the amino acid composition of these preparations have the same constituents, the mole ratios were not identical. They attribute these differences to a core peptide with "ragged edges" due to the method of degradation.

c) ELECTROPHORETIC MOBILITY: The somatomedins have been separated by cellulose column electrophoreses (7), isoelectric focusing (8) and polyacrylamide gel electrophoresis (1). Somatomedin C has been electrophoretically separated using 15 percent polyacrylamide gel at a pH of 2.3 in 4 M urea. In this situation, its mobility is greater than insulin. Somatomedin A focuses at a neutral pH when subjected to a 2 percent ampholyte mixture containing 6 M urea at 5° C for 87 hours exposed to approximately 300 volts.

d) ISOELECTRIC POINT: The data of Van Wyk and associates (1) suggest three isoelectric points for the somatomedins. They are approximately pH = 4.0, pH = 7.0 and pH = 8-9. They do not identify which peak represents which somatomedin. Somatomedin B is considered an acidic peptide and probably will be found at the pH = 8-9. Somatomedic C is the basic peptide and will therefore be recovered at the lower pH peak.

TABLE I

AMINO ACID RESIDUES IN SOMATOMEDINS

	Somatomedin A	Somatomedin B
Aspartic	5	5
Threonine	2	4
Serine	3-4	3
Glutamine	5	8
Proline	7	1
Glycine	7	2
Alanine	7	1
Cysteine	1	8
Valine	5	2
Methionine	1	-
Isoleucine	1	-
Leucine	3	1
Tyrosine	1	3
Phenylalanine	2	1
Histidine	4	-
Lysine	2-3	4
Arginine	5	1
Tryptophan	-	-
	60	44

e) SOLUBILITY: The somatomedins are soluble in acid-ethanol at 5° C, 20% formic acid, 6 M urea, 0.05 M N-ethyl morpholine acetate buffer, 0.05 M pyridine acetate buffer, 7.8% acetic acid - 2.5% formic acid, 0.02 N hydrochloric acid, Waymouth's medium MB - 752/1 with 1% albumin (9), tris - HCl buffer at pH = 7.4 (2) and 0.02 M barbital buffer. When somatomedins A and B are dissolved in 1 M acetic acid, they are partially separated from their carrier protein (2). They are insoluble in an acetone: alcohol mixture at - 15° C. They can be removed from plasma in the Cohn fraction IV b by salting out (1). It is stable to heat in the range of 60-100° C (20). It is destroyed by trypsin and partially denatured by mercaptoethanol (20).

f) SPECIFIC GRAVITY: None has been reported in the literature to our knowledge.

g) TISSUE CULTURE: Somatomedin is produced in the liver. To our knowledge the specific cells producing somatomedin have not been identified nor has any liver cell tissue culture been established to produce this hormone. A number of studies however, have involved the regulation of somatomedin generation in the isolated perfused liver (9, 10, 11, 12).

h) METHOD OF TISSUE CULTURE: See section g. If it is found that hepatocytes are the cell source for these hormomes, the procedure for tissue culturing hepatocytes (13) can be found in the section on Alpha-1-Antitrypsin, pages A-13 and A-14 of this volume.

i) TECHNIQUE OF ASSAY: This class of hormones had as its original focal point the fact that they could cause the uptake of sulfate into cartilage. A number of in vitro bioassays were developed for this purpose. When it was discovered that some "sulfation factor" preparations could affect other physiological parameters sometimes but not all the time, it became common to test for the sulfation uptake as well as another parameter. This led to the discovery of three distinct somatomedins by bioassay technique.

Somatomedin A designates the hormone that stimulates incorporation of sulfate into chick cartilage. Somatomedin B increases DNA synthesis in glial cells. The factor enhancing both sulfate and thymidine uptake into rat cartilage is somatomedin C.

The classic hypophysectomized rat costal cartilage segment assay (14) is technically demanding and the least precise of the three methods. The 12-15 day embryonic chick pelvic rudiment assay (3) is easier to perform and quite precise but it is not as sensitive as the rat assay nor can it measure thymidine incorporation. The porcine costal cartilage disc assay (15) is technically easy, very precise and allows multiple samples in a single assay. All these biological assays respond to an insulin-like action of other biological materials as well as to stimulatory and inhibitory factors in serum. It is thus often difficult to decide whether a decrease in the biological activity of serum is due to a decrease in stimulatory factors or an increase in inhibitory factors.

The availability of purified somatomedin A and B has led to the development of more specific and precise methods for their determination, such as a radioreceptor assay for somatomedin A (16) and a radioimmunoassay for somatomedin B (4). The high concentration of salt in urine sometimes interferes with the determination of somatomedin A by the radioreceptor assay. The labelled somatomedin A binds specifically not only to chick cartilage membranes but also to membranes prepared from a variety of rat and monkey tissues such as lung, kidney, liver, brain, thymus, spleen, pancreas, heart and fat (17). The labelled somatomedin B does not bind to any membranes prepared from rat and monkey tissues (2).

EXPECTED SPACE IMPROVEMENTS: Various forms of electrophoresis are currently used on the ground in conjunction with other isolation procedures. Unfortunately their yield is very low and the products are denatured or fragmented in the process. Space processing of the concentrated material would provide a method of increasing throughput as well as yield while also extracting other products at the same time.

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NAME (GENERIC): TRANSFER FACTOR

(PROPRIETARY): NONE

DISEASE TREATED: Multiple Sclerosis
Leprosy (Hansen's disease)
Malignant Melanoma

PATIENTS: Multiple Sclerosis is the most common neurological disease next to cerebrovascular accidents (strokes). Mr. Allenby, executive director of the St. Louis Chapter of the Multiple Sclerosis Foundation in Missouri reports that this disease affects about one of every four hundred adults in the United States or approximately 535,000 people. It is a chronic, slowly progressing disease of the central nervous system characterized by scattered patches of degeneration within the myelin sheaths of nerves in the brain and spinal cord. This degeneration usually leads to a number of symptoms including weakness or loss of function of one or more limbs, visual, vocal and mental disturbances and loss of bowel and bladder control. The disease is not lethal of itself but weakens the body over a period of time making it susceptible to secondary infections, etc. In two-thirds of the cases of this disease, the onset of symptoms occurs between the ages of 20 and 40. Although the average length of life is 10-15 years after onset, many patients live much longer. Some patients have frequent attacks and are rapidly incapacitated while others have periodic remissions for as long as 25 years. There is no specific therapy for this disease and spontaneous remissions make any treatment difficult to evaluate. The cause of the disease is unknown but recent positive responses to transfer factor suggest it may be the result of a viral or bacterial infection requiring a long period of time for incubation.

Leprosy or Hansen's disease is a chronic, possibly mildly contagious disease caused by a mycobacterium characterized by both local cutaneous and constitutional symptoms as well as the production of various deformities and mutilations. Over the last eight years the National Center for Health Statistics reported approximately 130 new cases per year in the United States. The disease is endemic in the gulf states in America, Hawaii, Puerto Rico and the Philipines and is found in the tropical and subtropical portions of Asia, Africa and South America. In these

tropical countries it can affect as many as fifty people per thousand population. The onset of the disease is insidious with gradual development of skin lesions, nodular development, neurological disturbances and anemia. The prognosis depends on the extent and character of the lesions. Spontaneous remissions frequently are encountered in the tuberculoid type of the disease. After an average period of eighteen years, death usually ensues from an intercurrent disease or asphyxiation secondary to occlusion of the glottis by mycobacterium nodules. Usually patients with this disease are isolated.

Malignant melanoma strikes about 14,000 men and women each year in the United States. It accounts for most of the 5,900 lives lost annually to skin cancer. In Australia, the number of people presently with malignant melanoma is sixteen per hundred thousand population, the highest ratio for this disease in the world (23). Starting as a dark mole on the skin, the malignancy spreads rapidly under the surface of the skin and requires extensive surgery to remove the draining lymph nodes in order to limit the spread of the cancer. In many cases where the disease is advanced or the lymphatic drainage is uncertain, recovery is poor.

Since Transfer Factor (TF) is one of the substances responsible for cell mediated immunity it will have therapeutic applications in many areas where the natural immune system of the body is deficient or lacking. Such conditions include asthma, atopic dermatitis, severe disseminated fungal infections, osteogenic sarcoma, and carcinoma of the bladder.

SEVERITY ESTIMATE: People afflicted with these diseases run the gamut from chronic debilitating problems to a terminal condition. In malignant melanoma the condition may lay dormant for years and flare up rapidly, resulting in death in a very short time. Lepromatous patients become disfigured as the disease progresses. Historical prejudice and ignorance of this mildly contagious disease have relegated patients to isolation from the regular population. Multiple sclerosis is a crippling disease that has sporadic flare ups and remissions but usually leads to eventual dependence on other people for existence. A person known to this group has been afflicted with this disease for forty years.

TREATMENT REGIMEN: Multiple Sclerosis - 0.1-0.2 units of TF at weekly intervals subcutaneously depending on patient response.
Leprosy - 7.4 units of TF given in 3 divided doses over a 12 week period.
Melanoma - 1.0 unit of TF subcutaneously in the abdominal wall at biweekly intervals.

TREATMENT METHOD:

Multiple Sclerosis - Angers and his associates (23) studying twenty patients with Multiple Sclerosis have found that the return of normal bladder function in six patients appeared within two or three days after administration of the first dose of TF. One 25 year old patient with limping and generalized weakness had complete disappearance of all symptoms after one treatment with 0.2 unit of TF. While receiving monthly injections at the same dose, no exacerbations of her condition presented during the remaining ten month period of the study. Nine patients exhibited a good partial response to TF exhibiting a disappearance of muscle weakness, improvement in motor coordination and a sense of well being. One of these patients had a return of hand movement after ten years of near immobility. The dosage requirement for each patient appears to be variable and erratic for maintenance of an improved state.

It should be noted that this disease is subject to spontaneous remission for varying periods of time. The prolonged improvement in these patients may be due to this remission state.

Leprosy - Hastings and his associates (24) observed clinical improvement in all five of the patients they were treating. There were significant reductions in bacilli seen in skin scrapings taken from six sites on each patient at weekly intervals. This clearing rate was six times faster than that observed with the standard dapsone chemotherapy. They did note that the clinical intensity of the reversal reaction in four of their patients peaked in intensity during the 6-9th week of TF therapy. They suggested that the mechanism of this immunosuppressive effect of TF might include: a) massive destruction of the bacilli with release of large quantities of desensitizing antigens, b) the development of, or increases in, serum blocking factors such as antigen-antibody complexes and c) the induction of specific suppressor "T" cell activity.

Melanoma - In a study of the value of transfer factor immunotherapy in cancer (25), Vetto and associates observed melanoma regression in three of eleven patients when TF was administered in a single dose. One of the patients showed complete remission for the full twelve months of the study. In two of these remission cases the tumor reappeared after five and three months, respectively. In these remission patients and the eight others showing no response to treatment, TF administration was maintained at biweekly intervals. Other investigators (26) were unable to find any remission for melanoma when one unit of TF was administered every three to four days for a total of twenty-two doses.

CURRENT GROUND STATUS: That human white blood cells could effect a cutaneous delayed hypersensitivity to an antigenic type material was first demonstrated by Lawrence in 1949 (14). Further investigations led him to the observation that extracts of these leukocytes were as effective as viable cells in the transfer of this delayed hypersensitivity resulting in the concept "transfer factor" (16). Both the cells and the leukocyte extract require up to 96 hours after injection into a patient to demonstrate this hypersensitivity both locally and systemically when challenged.

Transfer factor is unusual in that it confers this immunity for long periods of time, greater than two years in some instances, as compared to most antigen-antibody reactions which last only several days to several months. It is believed that this transfer factor induced delayed type of immunity will last as long as the cell source is still viable within the patient or in vitro tissue culture. The cell source can be a previously sensitized cell transplanted into the patient. An alternate source is the patient's own cells induced to produce additional transfer factor upon in vivo exposure to subcutaneously injected exogenous dialyzed transfer factor.

It is still not known if transfer factor is a single entity or a class of closely related substances. It is believed to be a polypeptide-polynucleotide complex that is different from interferon in chemical properties and biological action. Several methods have been used for the isolation and purification of this product,

including various forms of electrophoresis, chromatography, and dialysis. A contaminant free, single fraction of this substance has not been isolated to date.

The American Cancer Society is currently funding Lawrence to develop an in vitro assay for transfer factor.

The clinical effects of transfer factor are currently being explored in many pathological conditions including breast cancer, Wiskott-Aldrich syndrome, immunodeficiency disease, viral infections, hepatitis (2). In most of these studies only preliminary results on a small number of patients have been obtained. Additional studies will have to be completed before decisions can be made about the biological efficacy of transfer factor against these diseases.

This product is not commercially available at the present time and is not expected to be in the next five to ten years. Most laboratories, using transfer factor in clinical trials, make their own material from the blood of donors related to the patient. There are no substitutes for this product for most responding diseases. However, leprosy is presently being treated with dapsone. Radiation therapy and chemotherapy are used with some success against cancer.

b) STARTING MATERIAL AND PRODUCTION METHOD: Transfer Factor is the active biochemical agent present in a particular population of circulating leukocytes found in specifically sensitive human subjects. This agent transfers cutaneous reactivity of the delayed type to nonsensitive individuals. It has been shown that "T" lymphocytes or monocytes alone are sufficient to transfer sensitivity. This general immunological phenomenon has been established for a variety of bacterial, fungal, viral, denatured-protein and histocompatibility antigens.

A Transfer Factor for a specific immunological agent can be obtained from circulating human leukocytes in the following ways: a) concentrates of viable leukocytes (14), b) leukocyte extracts prepared by distilled water lysis or by repeated cycles of freeze-thawing (15), c) leukocyte extracts prepared by treatment with

enzymes, such as DNASE, RNASE, DNASE + Trypsin (16), d) antigen liberation (17) and e) dialysis (18). Lawrence and Al-Askari describe these methods of preparation in great detail with the advantages and disadvantages of each (19).

In each of the above sources for TF, the preliminary step has been the isolation and concentration of leukocytes from human plasma. Blood donors must be selected on the basis of demonstrating an intense degree of delayed cutaneous reactivity to the specific antigen under study. Using sterile procedures, venous blood from the ante cubital vein is drawn into a Fenwal bag equiped with a cation exchange resin to prevent coagulation. To facilitate erythrocyte sedimentation, dextran (4 mg/ml blood) is added to the bag and mixed gently but thoroughly. The bag is suspended in an incubator at 37 deg C for 30-45 minutes until the erythrocytes have sedimented leaving a plasma-leukocyte supernatant with a yellow ground-glass appearance. If the supernatant has the clear appearance of serum and a buffy coat of leukocytes appears on top of the erythrocytes, the leukocyte yield will be markedly reduced. The erythrocytes are drained from the bag by the plastic tubing provided and then discarded. The leukocyte-plasma mix is removed from the bag through the same tube and collected in 10 ml aliquots within special conical tubes calibrated at their tip in hundredths of a ml. (These "Lawrence Tubes" are available from Fisher Scientific Company). The capped tubes are centrifuged at 1,000 RPM for 10 minutes in a trunion ring centrifuge. The cell free plasma is decanted. The leukocytes appear as a layer above any contaminating erythrocytes at the bottom of the tube. Fifty microliters of sterile, pyrogen-free saline is gently layered over the leukocytes. A Pasteur pipette is inserted through the saline and the leukocyte-saline layer is removed by suction. Since mechanical injury to the cells causes leakage of TF into the surrounding fluid, minimum quantities of fluid should be used for resuspension. Each 0.1 ml of packed leukocytes isolated by this technique is equivalent to approximately 8.5×10^7 cells. One unit of TF is considered equivalent to 8.5×10^9 cells or the equivalent of the extract from that quantity of leukocytes.

The collected leukocyte-saline mixture from each tube is pooled into one 1.6 x 12 cm Pyrex test tube and frozen in an alcohol-dry ice mixture until a nipple forms at the center of the meniscus indicating a solid freeze. The tube is then placed

in a water bath at 37°C and thawed to a liquid or gelatinous consistency depending on both the lymphocyte concentration and amount of endogenous DNASE released and activated at this temperature. This freeze-thawing procedure is repeated for nine additional cycles and results in complete disruption of the leukocytes. The TF activity is now located mainly in the supernatant but some activity is also present in the cell sediment.

DNA is removed from the leukocyte extract by adding 1 mg of pancreatic DNASE and 1 mg of magnesium sulfate to the solution. This can be done at any time during, or after, the first freezing cycle. This enzyme treatment depolymerizes all the extracellular DNA liberated by the freeze-thawing process and converts the leukocyte extract from the consistency of suspended mucus clumps to a watery liquid state.

The clarified leukocyte extract is then placed in a 0.62 mm diameter cellophane dialysis tubing, taking care not to contaminate the exterior surface. The filled tube is then placed in a sterile, pyrogen free physiological saline bath containing fifty times more saline than the volume of fluid within the dialysis tubing. The dialysis is carried out for eighteen hours on a shaker table in a cold room. The TF will dialyze through the membrane wall into the surrounding fluid freeing it from the contamination of most of the higher molecular weight proteins.

The dialyzate containing transfer factor is pipetted into a series of 500 ml sterile lyophilization bottles. After the bottles are shell frozen in an alcohol and dry-ice bath, they are attached to a lyophilizer for 12-18 hours. The resulting whitish-yellow powder is collected, sealed and stored under ordinary refrigeration until ready for use in animal or human clinical investigations.

Kahn and associates (7) have subjected the dialyzed TF to further purification using high pressure liquid chromatography. The column was packed with glycerol coated, controlled pore glass beads. The 0.375 inch diameter column was eight feet long. Elution was carried out with 0.05 M sodium chloride solution. Their second of six fractions gave two bands on thin layer chromatographic analysis. The bands are currently being characterized for amino acid content.

Burger and co-workers (10) as well as Lawrence (2) have described a purification technique using exclusion chromatography in an ascending column (2.5 x 90 cm) packed with Sephadex G-25. The dialyzed transfer factor is eluted with distilled water pumped at 30 ml/minute at 4 deg C. The resulting peaks have been subjected to isoelectric focusing for additional purification and biochemical investigation.

The resulting product from each of the three methods has been used for animal and human pharmacological trials. While sterile procedures are required throughout each process, the final product must be passed through a Swinney filter prior to injection into a patient. This filtration is necessary to clear the solution of any possible bacteria introduced from the outside of the dialysis tubing during preparation of the dialyzate because the dialysis tubing can not be sterilized.

c) YIELD: The actual yield for each procedure has not been reported. It is known, however, that the cell "ghosts" still retain some TF activity after the freeze-thaw cycling. The cellophane tube used for dialysis will also retain some TF which can be removed by a second and third exposure to a dialysis bath. It is known that the TF extract from 83 x 10^6 cells will be sufficient to induce systematic delayed hypersensitivity to a challenging substance. This quantity of packed leukocytes can usually be found in 0.1 ml of the plasma-leukocyte supernatant obtained from 10 ml of white blood. Foster reported that TF contains 1 mg of peptide and 300 μg RNA per 10^9 lymphocytes (1).

d) PURITY: The chemical composition and molecular structure of TF has not been determined. It is not even known if it is a single substance or attached to a carrier protein or nucleotide. The currently available, pharmacologically active TF must be considered impure and "contaminated" with unknown chemical constituents. The percentage of impurities present in the final product can not be determined at this time.

e) COST/DOSE (to patient) AND AVAILABILITY: Transfer Factor is not available commercially for research purposes or clinical investigation. At the current time, all TF is produced in the laboratories of the research scientists intending to use it.

f) STORAGE: TF in whole leukocyte preparations is stable in the deep freeze at -20°C for at least four years (11, 12). Dialyzed TF can be lyophilized and stored for at least 5 years at 4 deg C without loss of potency (11). Once cells containing TF have been put in saline or plasma and warmed to 37°C, the TF will leak out into the surrounding fluid and the cells will lose their sensitivity (13). Rough handling of the cells can also produce the same effect. When TF is heated to 56°C and held there for 30 minutes, it becomes inactive (11).

TECHNICAL DATA

a) MOLECULAR WEIGHT: Between 3,500 (1) and 10,000 (2).

b) MOLECULAR STRUCTURE AND AMINO ACID SEQUENCE: Although the precise biochemical nature of TF remains to be established, the search has been narrowed to a polypeptide-polynucleotide complex. It is known to contain ribose and peptide concentrations (3) based on wet chemistry analysis and ribose-nucleic acid (4). O'Dorisio and co-workers have reported that hypoxanthine is the major component of an affinity chromatographically prepared TF (5). Krohn and his group (4) have identified uracil as the major component of TF using a sephadex chromatography column. The presence of both or either of these purine and pyrimidine substances has not been confirmed by other investigators. It is believed by some that these substances may be degradation products of any nucleotides present, resulting from the method of isolation and characterization (6).

When dialyzed TF was subjected to high pressure liquid chromatography, six fractions resulted (7). Fractions 2 and 4 showed E-rosette formation with lymphocytes and were subjected to amino acid analysis. Each of these immune response fractions, identified as immunopeptides 1 and 2, have their amino acid residues listed in Table I. Note their minimal molecular weights of 1953 and 1427 for immunopeptides 1 and 2, respectively. Twelve different amino acids are present in immunopeptide 1 as compared to only ten in the second immunopeptide. Tryptophan and proline are the missing acids.

It appears that there are different polypeptides present in the transfer factor with different immunological activities. The seven components of TF isolated by Khan and associates (7) are currently being studied for chemical composition. It

TABLE I

AMINO ACID ANALYSIS OF TRANSFER FACTOR

Amino Acid	Immunopeptide Residues #1	Immunopeptide Residues #2
Lysine	1	1
Histidine	1	1
Arginine	?	?
Aspartic	1	2
Threonine	1	1
Serine	2	1
Glutamine	3	2
Proline	1	0
Glycine	4	2
Alanine	1	2
Cysteine	0	0
Valine	2	1
Methionine	0	0
Isoleucine	0	0
Leucine	1	1
Tyrosine	0	0
Tryptophan	1	?
Phenyl Alanine	0	0
Minimum Molecular Weight	1953	1427

is not known if these polypeptides are independent of one another or are active breakdown products of a larger moiety.

c) ELECTROPHORETIC MOBILITY: Transfer Factor previously subjected to adsorption chromatography has been subjected to electrophoretic separation using a 10% polyacrylamide gel (5, 8) with a tris-EDTA-Borate buffer at pH = 8.3. A single peak appeared near the cathode with a large diminishing tail toward the anode (5). This same electrophoretic pattern was observed with SDS electrophoresis (5) accomplished according to the method described by Weber and Osborn (9).

Using isoelectric focusing on TF previously treated by exclusion chromatography (10), at least seventeen subfractions were separated from the chromatography fraction III. Table II presents the isoelectric points observed with four different pH gradients ranging from 3.5-10. Biological activity appears to be localized in the subfractions with an isoelectric point lower than 4 according to Burger and his co-workers. Ampholytes appear to be a problem clinically with this product. They must be removed from the TF using exclusion chromatography prior to pharmacological testing.

d) ISOELECTRIC POINT: See section c, above.

e) SOLUBILITY: Transfer factor is soluble in distilled-deionized water, 0.05-0.15M saline, plasma, serum and tris-EDTA-Borate buffer at pH = 8.3. It is also soluble in ammonium acetate and 50 mM ammonium carbonate at pH = 7.4.

f) SPECIFIC GRAVITY: Unknown.

g) TISSUE CULTURE: Transfer factor is ordinarily prepared from the supernatant of a human lymphocyte culture. Such a preparation confers whatever cellular immunity the donor possessed to the recipient. In an alternative method, Pizza and his co-workers (20) have induced TF production in lymphocytes with formalin treated tumor cells (transitional cell carcinoma of the bladder). After induction, these lymphocytes were cultured for four or five weeks to meet their clinical investigatory needs. The authors warn, however, that the induction attempts were only eighty percent successful. These cultures, producing TF, can be cut back to one percent

TABLE II

ISOELECTRIC FOCUSING OF HUMAN TRANSFER FACTOR EXCLUSION CHROMATOGRAPHY FRACTION III

Gradient pH = 3.5-10	Gradient pH = 2.5-4	Gradient pH = 4-6	Gradient pH = 5-7
1-4*	1.95	1.1	1-5
5-7	2.3	3.7	5.85
7-8.5	2.9	4.05	6.2
10.2	3.1	4.30	6.75
	3.5	4.50	7.1
	3.6-8	4.65	
		4.80	
		4.95	
		5.8	

* Isoelectric point

of the final cell concentration at the end of three to five weeks and recultured for another TF harvest without using another induction period.

h) METHOD OF TISSUE CULTURE: The method of lymphocyte tissue culture for TF production used by Pizza and co-workers (20) is as follows:

1) Harvest leukocytes or lymphocytes by standard methods (19).
2) Suspend lymphocytes in 100 ml of TC 199 culture medium at the rate of 4-5 x 10^5 cells per ml.
3) Add dialyzed TF for a specific antigen to the culture in a quantity equivalent to that produced by 5 x 10^7 lymphocytes. This material is added to the culture by passing it through a 0.22 micron Millipore filter.
4) The cells, culture media and TF inducer are placed in a carbon dioxide, humidified incubator set at 37°C for 24 hours.
5) At the end of the incubation the cells are washed twice with TC 199 medium containing five percent fetal calf serum.

6) The cells are resuspended in 100 ml of the TC 199 medium containing five percent fetal calf serum and placed back in the incubator.
7) The culture medium is changed as required (usually twice per week).
8) The cultures can be cut back to one percent by harvesting approximately 10^8 cells each week.
9) Transfer Factor can be extracted from the harvested cells by the techniques described in the section on starting material and production method of this approach.

i) TECHNIQUE OF ASSAY: The most sensitive assay for TF at the present time is done in vivo on humans showing a negative reaction to a specific antigen when they are challenged by that antigen. If this negative recipient is first primed with an intradermal dose of TF prior to receiving the antigen, he will show a typical wheal or inflamation of the challenge site within 24-72 hours. It is only recently that this assay method has been adapted to animals (21).

In vitro assay techniques have been besieged with problems and reflect the detection of new receptors on the surface of the cell. Both leukocytes and macrophages are inhibited from migration in the presence of antigen when TF is present (2). Lymphocytes from immunodeficient subjects will form rosette patterns with erythrocytes in the presence of TF (7). Antibodies have recently been developed for human TF in rabbits (22) promising the opportunity for more definitive in vitro assay techniques.

EXPECTED SPACE IMPROVEMENT: Any purification of this material in large quantities would serve two purposes. It would provide the opportunity to biochemically characterize this product and large scale clinical trials could be undertaken to determine its effectiveness against a number of pathological conditions. While isoelectric focusing has been used as a final step in isolating small quantities of TF and representing the material in a number of bands, this technique does not have the capability of large scale production required to meet the two purposes. Only a continuous flow electrophoresis system operating in a zero gravity environment presents the opportunity for large scale throughput in the foreseeable future.

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NAME (GENERIC): UROKINASE

(PROPRIETARY): ABBOKINASE (Abbott Laboratories, Chicago, Illinois)

WINKINASE (Sterling-Winthrop Laboratories, Rensselaer, New York)

UKIDAN (Serono Laboratories, Boston, Massacheusetts)

DISEASE TREATED: Existing Blood Clots

PATIENTS: In clinical medicine, there are many potential uses for urokinase. These include: a) dissolution of arterial and venous thrombi anywhere in the body, b) lysis of pulmonary emboli regardless of source, c) liquefaction of intracardiac thrombi causing or the result of heart attacks and impairing prosthetic valve function and d) rapid resolution of certain inflammatory tissue reactions. Myocardial infarctions affected about one million people in the United States in 1975 resulting in 642,719 fatalities (25). A European Collaborative Study (26, 27) has been going on for a number of years to investigate the potential of urokinase as a means of minimizing heart damage within the first few hours after a heart attack. The American National Heart and Lung Institute has sponsored a clinical evaluation of the use of urokinase in the lysis of pulmonary thrombo-emboli (28). In England pulmonary embolism results in at least 2,000 deaths annually (29). An estimated one percent of the U.S. population annually is subjected to surgery, broken bones and accidents damaging the vascular system. As a result of this tissue insult, and the following immobilization, these people are all potentially subject to blood clot formation and its movement into the lungs.

SEVERITY ESTIMATE: The effect of blood clots can run the gamut from subclinical to clinical disability and death. When blood clots form within the veins, especially the veins of the legs or pelvis, there is a high risk of breakoff pulmonary embolism, which can be fatal. Another effect of intravenous thrombosis is impaired venous drainage of the affected body part, which commonly results in edema and ulceration of the legs. If the clot blocks minute vessels of the brain, physical and psychical changes can take place without the patient or his immediate family being aware of it.

TREATMENT REGIMEN AND METHOD: The objective of the treatment is to both dissolve the clot as soon as possible and provide prophylactic dosage to preclude further clot

formation until the patient is capable of near normal function. The recommended continuous intravenous infusion dosage for dissolving existing pulmonary clots was established at 4400 CTA (Committee on Thrombolytic Agents) units per kilogram of body weight per hour for 14 days. The total quantity of urokinase and heparin administered and duration of the urokinase therapy may vary with the location and severity of the clot but it is believed that a Lee-White Clotting Time of greater than 25 minutes should be used as a criterion for optimum therapy rather than dosage itself.

Edwards (29) applied urokinase directly to an area containing a pulmonary clot by using a balloon catheter to inject the material and retain it against the clot. 300,000 Plough units (one Plough unit is equivalent to about 1.5 CTA units) of urokinase were administered over a two hour period with noticable patient improvement within 30 minutes.

The European Collaborative Study established a treatment regimen for myocardial infarction at an initial dose of 7200 CTA units of urokinase per kilogram body weight given intravenously in 10 minutes followed by a maintenance dose of 3600 CTA units per kilogram per hour over a period of 18 hours (27).

Like all fibrinolytic materials, urokinase has an inherent problem in that it can cause hemorrhaging from old wounds when used at effective therapeutic doses (28). deTakatas (31) thought the hemorrhaging observed in the National Pulmonary Embolism Study was the result of overdosing with heparin.

CURRENT GROUND STATUS: Urokinase has been undergoing clinical trials for at least ten years. Its initial potential as a fibrinolytic or clot dissolving drug was as a replacement for streptokinase whose deleterious side effects forced the latter off the market. Urokinase is highly specific as an activator of plasminogen. Being of human origin, it is probably only mildly antigenic, if it shows any immune response at all. This will allow repetition of doses without development of resistance to its pharmaceutical action. Urokinase also requires only about half the quantity of product to achieve the same lytic effect produced by streptokinase. Both products, however, have the side effect of causing generalized hemorrhaging if the patient has any incompletely healed injury.

Urokinase has competition from therapeutic regimens employing cheaper, more widely used products. However, these products differ from urokinase in that they cannot dissolve existing clots but only prevent the formation of new ones. Current urokinase therapy in the United States indicates that urokinase treatment should be followed by one of these products (heparin) for at least fourteen days.

At the present time Sherry (29) feels it is difficult to document whether urokinase has virtues over streptokinase, other than for its non-antigenicity and freedom from those adverse reactions peculiar to streptokinase. For example, if urokinase and streptokinase were almost always successful in completely or extensively dissolving an occluding thrombo-embolus and restoring the circulation to normal or near normal, rather than a success rate of about 50 percent, there would be much less physician hesitation to use these products for initiating the treatment in all clotting episodes.

Three companies, Abbott Laboratories, Serono and Sterling-Winthrop, filed Investigational New Drug (IND) forms with the U.S. Food and Drug Administration in the late 1960s. In April of 1978, Abbott Laboratories was licensed to manufacture and sell urokinase under their brand name of Abbokinase. A recent telephone communication to the medical staff of Sterling-Winthrop has indicated that they do not expect New Drug Application approval for their brand of urokinase through next year. Serono has withdrawn its application in the United States but is still marketing the product in Europe. A Japanese company, Green Cross Corporation, has marketed the substance in its own country with 1977 sales reported to be 40 million dollars.

Unfortunately, the clinical investigation of urokinase has been hampered by the high cost of its production and relative lack of availability, whether prepared from cultured kidney tissue or extracted from collected urine. Twenty-three hundred liters of urine will provide only 29 mg of urokinase, about half of one treatment for one individual.

b) STARTING MATERIAL AND PRODUCTION METHOD: Urokinase can be obtained from two different biological starting materials, human urine and cultured kidney cells. At the present time the only urokinase preparation licensed in the United States is prepared from cultured kidney tissue, but some urokinase preparations marketed abroad are urine-derived.

Using human urine as the raw material for urokinase production, considerable difficulties were encountered because of the general variability of both the urine chemical composition and the concentration of urokinase present. An initial concentrating step was developed to reduce the massive volumes of urine required to collect a reasonable amount of urokinase. Guest and associates (1) successfully solved the problem by pooling the urine, and adjusting the pH to 4.3 in the cold. The resultant precipitate reportedly contained at least 90% of the original urokinase activity. Celander and associates (2) were able to recover about 72% of the initial urokinase activity in urine by the simple process of creating a foam by means of shaking, agitation or aeration. The foam is created by proteins forming surface tension films that can be harvested and collapsed back to the liquid state creating a twenty fold increase in urokinase concentration. Liquification of the foam is accomplished by adding 1-octanol to the foam in a concentration of 0.1 ml per 30 liters of urine.

A five step preparative scale purification procedure was developed by White and Barlow (3). It consisted of concentrating the urokinase in urine by foaming, cold (10°C) ammonium sulfate fractionation of the harvested foam, ion exchange chromatography on Amberlite IRC-50 and two gel filtration steps through Sephadex G-100. The resultant material gave two active materials that could be demonstrated by polyacrylamide disc electrophoresis and immunochemical techniques.

An alternative purification method was described by Lesuk (4). This involved a nine step process starting with precipitation of the urokinase by acidification of the urine. The redissolved precipitate was then adsorbed on to bentonite, eluted from the bentonite with 6, 9-diamino-2-ethoxyacridine lactate monohydrate, further adsorbed onto calcium phosphate gel and then eluted with sodium phosphate buffer. The eluate was treated with carboxymethylcellulose, then heat-treated and further absorbed and eluted twice with carboxymethylcellulose. The resultant material yielded a crystalline urokinase preparation.

A third method of purification by the use of affinity chromatography has been described by Pye and associates (5). Starting with a concentrated urokinase obtained by the above mentioned procedures of sequential foaming, salting out fractionation and then dialysis to remove the salt, the material was applied to a column of α-N-Benzylsulfoyl-p-Aminophenylalanine-Sepharose 4B Gel (BAPA). The

urokinase was eluted from the column using a solution of 8% sodium chloride. The urokinase yield was 100% of the original material applied to the column but the purity was increased only 130 times. Use of 1 millimolar BAPA as an elution material resulted in about 1300 times purification but only 50-60% of the original urokinase starting material. Other eluting agents have been used but were found to be far less successful than the 8% sodium chloride. These eluting agents included 4M urea, 6M guanidine - HCl and 0.1M sodium acetate buffer at pH of 4.5.

The recovery and purification of what is believed to be urokinase from the collected tissue culture medium of human kidney cells presented a technically more difficult problem because of the large diversity of other proteins in the growth medium (5). Since most of the protein content of culture media for this type of cell is albumin, it was readily removed by extraction on an oleic acid-Sepharose 4B affinity chromatography column developed by Peters (6). This column permitted 85-100% of the "urokinase" material to remain in the eluate. This eluate was mixed with a 0.1% TRITON X100 detergent solution to decrease the amount of other non-specific protein binding when it was poured through the usual BAPA column. Extraction with 8% sodium chloride resulted in purification factors of approximately 700 and a specific activity between 50,000 and 70,000 CTA units per milligram of protein.

c) YIELD: The production method resulting in crystallization described by Lesuk and associates resulted in 29 milligrams of urokinase from 2300 liters of urine representing an overall yield of 24 percent with sodium chloride fractionation (7). The material was reported to have a specific activity of about 100,000 CTA units per mg of estimated protein. Affinity chromatography techniques using a BAPA-Sepharose 4B column eluted with either 8 percent sodium chloride or 1mM BAPA resulted in yields of 100 percent and 50-60 percent of the originally applied activity respectively (5), however the columns rapidly lost efficiency. The material was concentrated to specific activities of 50,000-70,000 CTA units per mg of protein. The ion exchange chromatography technique of White and Barlow (3) resulted in an overall yield of about 42 percent of the activity found in the original material and a potency of approximately 218,000 CTA units per mg of protein.

The highest concentration of non-crystallized urokinase per mg we were able to find in the literature was 92,000 CTA units per mg of protein available from Serono Laboratories, Boston, Massachusetts (5).

d) PURITY: Usually crystallization of a material is a method of determining the absolute purity of that material. Lesuk's urokinase activity of about 100,000 CTA units per milligram does not coincide with the 218,000 CTA units of lyophilized powder obtained by White and Barlow. The major impurities remaining from fractionation procedures are albumin, urokinase precursors, urokinase aggregates and salts. Sodium chloride near physiological strength, EDTA in about 0.1% concentration, traces of albumin or gelatin and carrier lactose can all be found as impurties in the urokinase currently available. These materials serve as stabilizers.

e) COST/DOSE (TO PATIENT) AND AVAILABILITY: Abbott Laboratories is marketing Abbokinase at $700 per million units for clinical use. Serono Laboratories will supply URIDAN (their brand of urokinase) in the United States at $400 per million units for research purposes only. Sterling-Winthrop has filed a New Drug Application for its brand of urokinase with the Food and Drug Administration but it has not been approved as yet. Discussions with the medical department at Sterling-Winthrop indicated that FDA approval was not expected next year. All three of these companies market the product in Europe along with Choay in France and have a Japanese firm giving them competition in the far-eastern market.

f) STORAGE: Urokinase is a moderately stable enzyme showing no appreciable loss in activity over years in the lyophilized state or over months in sterile solutions at 1 mg/ml or more when refrigerated (3). In dilute solutions it has been found advisable to maintain it in a concentration of 0.1 percent EDTA when being held at room temperature for more than a few hours. Abbott Laboratories currently packages their European product in 5 mg carrier lactose per 1 mg of urokinase.

TECHNICAL DATA

a) MOLECULAR WEIGHT: A number of investigators have described "purified" urokinase obtained by salt fractionation, ion exchange chromatography and affinity chromatography as forming two separate bands using polyacrylamide gel electrophoresis.

These bands, S_1 and S_2, had molecular weight of 53,000 and 33,100 with specific activities of 100,000 CTA units per mg and 218,000 units per mg, respectively (18). The urokinase crystallized by Lesuk (7) showed only one band with polyacrylamide gel

electrophoresis in several different pH solutions and was in agreement with the molecular weight and specific activity of the heavier band. Free flow electrophoresis of a commercial preparation (8) presented four bands of urokinase. The major and minor bands were common to the molecular weights of 53,000 and 33,100 but the two trace activity bands had molecular weights of 47,000 and 43,000. Schonebeck and associates (13) took Lesuk's original pooled urokinase material and subjected it to isoelectric focusing (pH = 3 to 10, 300 volts, ampholine solution and sucrose as an anticonvectant) for 72 hours. Two major peaks were formed. Their first peak (pH 4.2) was a high molecular weight material while the second, broader peak (pH = 9) had a high and low molecular weight material. They postulated that urokinase is either more than one substance or exists partly free and partly bound to protein or that the material has aggregated, producing peaks at different molecular weights. Studer and associates (8) believe the urokinase weighing 53,000 daltons represents native urokinase and the lower molecular weight material is the result of emzymatic degradation during storage of the urine. Bangham (9) as well as White and associates (3) and Johnson (10) on the other hand, believes the 33,000 dalton material is the native urokinase and the heavier weight material is a precursor or carrier.

b) MOLECULAR STRUCTURE AND AMINO ACID SEQUENCE: The major and minor bands have been analyzed by Edman degradation even though the heavier material could not be completely removed from the product tested. The molecule was greater than 90 percent protein and about 7 percent water with the balance as aminohexoses (8). Seventeen amino acids were present in the quantitative ratio seen in Table I. Tryptamine was not determined. The heavier product contained 469 amino acid residues while the lighter product contained 287 amino acid residues (10). Studer and associates were in close agreement with the quantitative and qualitative amino acid analysis of the lighter product (8). They also did additional work on the sequencing of these amino acids in the 33,000 dalton material. The N-terminal band can be defined as:

Ile	Ile	Gly	Gly	Glu	Phen	Ser/Thr(?)	Thr	Ile	Glu	Asp	Glu	Pro	Trp	Phe	Ala	Ala	Ile	Tyr
1	2	2	4	5	6	7		8	9	10	11	12	13	14	15	16	17	18	19

TABLE I

AMINO ACID COMPOSITION OF URINARY UROKINASE

AMINO ACID	51,700 MOLECULAR WEIGHT BAND	33,400 MOLECULAR WEIGHT BAND
LYSINE	35	19
HISTIDINE	23	10
ARGININE	25	15
ASPARTIC	43	24
THREONINE	28	20
SERINE	35	22
GLUTAMINE	48	33
PROLINE	30	17
GLYCINE	45	27
ALANINE	23	14
HALF-CYSTINE	20	9
VALINE	22	11
METHIONINE	8	5
ISOLEUCINE	18	15
LEUCINE	33	24
TYROSINE	19	12
PHENYLALANINE	14	9
TOTAL NUMBER OF AMINO ACIDS	469	287

for the first nineteen amino acid residues. The further sequencing depends on the availability of material.

c) ELECTROPHORETIC MOBILITY: Urokinase has been subjected to a number of electrophoresis techniques including sodium dodecyl sulfate (SDS) disc gel electrophoresis (5, 3), disc gel isoelectric focusing (5), and free flow electrophoresis (8). Lesuk reported that the degree of resolution obtained by the disc-electrophoretic analytical technique surpassed that obtained with other zonal (paper, cellulose acetate, starch gel) electrophoretic procedures (7). The crystalline material produced one band on gel electrophoresis while most other separation methods produced two or more bands as mentioned in section b. Polyacrylamide gel electrophoresis has been conducted at pH of 4.3 (7) and pH of 8.6 (8). The kidney cells producing urokinase have also been subjected to electrophoretic separation. Allen and associates (11) used a zonal electrophoretic technique with the cells suspended in phosphate buffer at 7.3 pH and an ionic strength of 0.0097 moles/liter. Kolin and Luner (21) had previously shown that these kidney cells could be fractionated using an endless belt electrophoretic technique.

d) ISOELECTRIC POINT: Pye and associates (5) reported urinary urokinase as having an isoelectric point for the more active band at 8.9 and about 4.5 for the minor band. "Purified" adult human kidney urokinase demonstrated an isoelectric point at 8.75. Using human fetal kidney "urokinase" and a moving boundary partition electrophoresis technique, Barlow and Lazar reported an isoelectric point of 6.9 (12).

e) SOLUBILITY: Urokinase is freely soluble in water. It is also soluble in mild acid solutions (pH 4 and above) if the solvent remains at room temperature. BAPA TRIS, and phosphate buffers, 1M KCL and 8 percent sodium chloride solutions can be used for elution of the product. EDTA in a concentration of 0.1 percent may have to be added to the buffer (3). Urokinase insoluble in alcohol, acetone, 98% sodium chloride saturation and 65% ammonium sulfate saturation. Lesuk reported that urokinase aggregates at a pH of 6.8 but this process is reversible and did not appear at any other pH value over the range of pH 2.1-10.5. The chloromethyl ketones denature urokinase.

Urokinase is a moderately stable enzyme showing no appreciable loss in activity over years in lyophilized form or over months in sterile solutions of 1 mg/ml or more at refrigeration temperatures (3). In dilute solutions it has been found advisable to maintain a concentration of 0.1 percent EDTA when dialyzing or holding for more than a few hours. Stability is decreased at salt concentrations below 0.03M sodium chloride and precipitation, with loss of activity, occurs at very low salt concentrations. White and Barlow advise adding a protein such as human serum-albumin, protein Fraction V or gelatin to prevent surface denaturation when urokinase is in very low concentration such as seen in fibrinolytic assay work. In 1964 Abbott Laboratories prepared material as an international standard for the World Health Organization Expert Committee on Biological Standardization that contained 5 mg of carrier lactose plus 1.5 mg urokinase extract.

Urokinase saturation levels in various solutions have not been reported in the literature as far as we can determine.

f) SPECIFIC GRAVITY: The specific gravity of the cells producing urokinase is not known to us.

g) TISSUE CULTURE: Ladehoff found that medulla of the kidney contained high concentrations of urokinase in 1960 (14). He suggested that this product could have been released from active desquamated cells in the urinary tract. Myhre-Jensen (15) confirmed that urinary tract surface epithelial cells are highly fibrinolytic. When Barnett and Baron (16) reported that certain strains of cells derived from kidney cell cultures yielded urokinase in their culture fluid, other investigators began to explore the use of such cultures as a source for urokinase (17, 18, 19 and 20). Barlow and associates (18) used human embryonic kidney cortex (10). Bernik and Kwaan have started cultures from human kidney cortex (20). Apparently the actual cells producing urokinase have not been identified in the kidney. This lack of knowledge led to the electrophoretic experiment aboard the Apollo-Soyuz Space Flight to try to fractionate human kidney cortex cells into a portion that produced urokinase (11). The spaceflight-separated cells were recultured when returned to earth. One urokinase producing cell was able to manufacture 80,000 CTA units (about one milligram of urokinase) in 35 days of production culture compared to 11,200 CTA units per ground control cell.

Barlow (18) found that there was essentially no development of karyological alterations through ten subcultures each lasting an average of 35 days. No feedback mechanism is evident to slow down or stop urokinase production when "purified" urokinase is added to the production medium. The addition of small amounts of trypsin also brings about a significant increase in the amount of urokinase produced by breaking down the lactalbumin hydrolysate of the production medium into dipeptides and amino acids for easier assimilation by the cells. This trypsin addition has also shown that urokinase is released from the cell into the culture media in precursor form. The trypsin will reduce it to its active form.

a) METHOD OF TISSUE CULTURE: Barlow and associates obtained human embryonic kidney cells from Flow Laboratories, Rockville, Maryland. These cells were grown to confluency in Eagles Medium (E-199) containing 10 percent fetal calf serum (as a growth medium) at 37°C in an humidified atmosphere of 5 percent carbon dioxide in air (18). The cells were grown in plastic tissue culture flasks having 75 square centimeters of growth surface covered by 40 milliliters of the growth medium. Confluency was attained in 7-10 days. (No mention was made of cell concentration at the start of culturing or at confluency nor were their any indications of the rate of media change during this time period.) Once this confluency was established the media was switched to 0.5 percent lactalbumin hydrolysate to serve as a production medium. He reported that the tissue culture process takes 30 days to achieve maximum activator production. Because his article reported each subculture contained 3-4 generations prior to switching to a production media in 2 to 10 days we are assuming that the rate of urokinase producing cell reproduction is on the order of about 54 hours.

Barlow and associates used these tissue culture conditions for growing the same type of cells in what they call a mass tissue culture propagator containing 16 liters of medium. This suspension cell culture system was described by Weiss and Schleicher (22, 23).

Maciag and associates (19) used adult pig kidney cells for their cultures. The cells were grown in Waymouth's MB752/1 medium supplemented with 3 percent serum and 1.5 percent antibiotic-antimycotic solution contained in collagen coated plastic tissue culture dishes initially seeded with 2.5×10^6 cells per 28 square centimeter

dish. The cells were fed daily with replacement culture media, subcultured every 7-10 days and grown in a 37°C humidified atmosphere containing 95 percent air and 5% carbon dioxide. They were able to substitute 2% bovine serum for 3% porcine serum in the culture media if needed.

i) TECHNIQUE OF ASSAY: There is as yet no pharmacopeial test devised which can show urokinase in a minimum specific activity. The standard assay currently in vogue is the Committee on Thrombolytic Agents fibrin plate assay described by Astrup and Kok (24). The Lee-White clotting time is also used clinically to show that clinical doses require at least twenty minutes for the blood sample to clot. Bangham suggests a series of tests (9) to develop a urokinase standard. This includes identification by: a) very high plasminogen activation potency, b) contains protein of only human origin by immunological testing, c) that it activates plasminogen of different species differentiating it from streptokinase and d) a specificity finger print making use of its particularly high affinity for certain substrates like the chromogenic tetrapetide H-D-Valine-Proline-Arginine-pNA (BOFORS #S 2234).

EXPECTED SPACE IMPROVEMENT: The weightless characteristic of space can be used to enhance the separation of urokinase-producing cells from other kidney cells. The ability to obtain large quantities of these cells to start tissue culture producing facilities in space or on the ground will greatly help to provide an adequate supply of the product as well as reduce its cost to a patient affordable level.

While free flow electrophoresis has been used to purify some urokinase on earth, the lack of gravitation in space could tremendously increase rapid production capability for purified product.

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